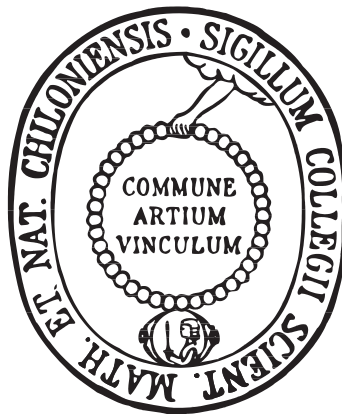


# **Marine Biofilm Formation Dynamics – From Sensor Development To Field Applications**

Dissertation  
zur Erlangung des Doktorgrades  
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der Christian-Albrechts-Universität zu Kiel



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**Dipl.-Ing. (FH) Matthias Fischer**

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*Meis dedicatum.*

Referent: Prof. Dr. Martin Wahl  
Korreferent: Prof. Dr. Gernot Friedrichs

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Der Dekan





# Abstract

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BIOFILMS represent one of the oldest microbial lives as they have been already formed on our planet about 3.43 billion years ago. In aquatic environments, nearly all submerged living and non-living surfaces become rapidly colonized by bacteria, fungi, diatoms, protozoa, invertebrate larvae and algal spores. Mature biofilms play an important role in nutrient cycle, food chain, decomposition of organic matter and subsequent colonization of higher organisms. The understanding of biofilm formation dynamics is of substantial biological interest. In this thesis, an optical fiber-based biofilm sensor has been developed in order to dynamically detect biofilm formation of bacteria and unicellular microorganisms in their natural marine environment. The device is based on the detection of natural fluorescence utilizing the intrinsic amino acid tryptophan of microorganisms constituting the biofilm. Promising sensor head geometries were modeled and optimized in terms of the spatial arrangement of the entire optical system and of the light emission and collection characteristics. Thereby it could be shown that the inclination angles of the optical fibers are crucial for surface sensitive detection of biofilms. The sensor head design is capable of detecting biofilms grown on a large surface of about 1 cm<sup>2</sup>. The intrinsic fluorescence originating from biofilms disposed on a UV transparent substrate is excited by a 280 nm UV-LED. The emitted fluorescence is collected and guided by optical fibers to a photomultiplier tube operating in photon counting mode. Interference filters are utilized to spectrally separate the 350 nm emission from background and scattered excitation light. Calibration measurements demonstrate that tryptophan can be detected in the nanomolar concentration range and at low biofilm coverages down to < 0.01%. A wide dynamic range enables the study of biofilm formation from the first attachment of cells up to complex and mature cell clusters. In field experiments, biofilm formation dynamics has been quasi-continuously monitored by exposing the sensor to Baltic Sea and Pacific Ocean waters over a period of several weeks. It has been discovered that the initial attachment of bacteria during the first hours is independent from the environmental conditions. In contrast, by monitoring microbial settlement over several weeks, seasonal variations in cell density were observed. Based on the *in situ* data in combination with epifluorescence microscopy, a mathematical model has been built to describe natural biofilm establishment and dynamics. Moreover, the quasi-continuous measurements

show diurnal variations of fluorescence intensity. By studying biofilm formation dynamics in close proximity to the macroalgae *Fucus vesiculosus* the growth rate of colonizing bacteria forming a biofilm is significantly higher. Bacteria from the class of *Alphaproteo* and its genera *Rhodobacter* and *Roseobacter* were the most dominant group on the settling substrate. On the surface of the macroalgae *F. vesiculosus*, *Alphaproteobacteria* were identified as the main colonizer. To identify chemical compounds that mediate abundance and community composition of bacterial biofilms, the effect of surface-available algal compounds has been studied under field conditions. The polar fraction of the algal surface extract revealed a significant profouling effect, whereas the non-polar fraction containing the xanthophyll pigment fucoxanthin and other unidentified non-polar surface compounds revealed a significant reduction of surface colonizing bacteria. Moreover, the analysis of bacterial surface communities by 454 pyrosequencing demonstrated that the antifouling activity of non-polar algal surface compounds was targeting the abundance of natural bacterial colonizers rather than the relative composition of bacterial members within the community.

# Zusammenfassung

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**M**IKROBEIELLE Biofilme besiedeln unseren Planeten seit ungefähr 3,43 Milliarden Jahren und stellen die älteste und die am weitesten verbreitete Wachstumsform dar. In aquatischen Systemen wie Ozeane, Flüssen und Seen werden alle lebenden und nicht-lebenden Grenzflächen von Mikroorganismen wie Bakterien, Mikroalgen, Pilzen, Protozoen besiedelt und bilden einen Biofilm. Daher kommt diesen eine herausragende Bedeutung in der marinen Ökologie zu, da sie zu einem großen Teil an Stoffwechselaktivität und biogeochemischen Prozessen beteiligt sind. Für viele Organismen stellen Biofilme die Nahrungsgrundlage dar und haben maßgeblich Einfluss auf die Folgebesiedlung lebender und nichtlebender Oberflächen. Es ist daher von großer wissenschaftlicher Bedeutung, die Bildung des Biofilms und deren Dynamik unter natürlichen Bedingungen kontinuierlich messen zu können. Zu diesem Zweck wurde ein optischer Feldsensor entwickelt, der ein quasi-kontinuierliches Messen der Biofilmdynamik im Meer ermöglicht. Die Eigenfluoreszenz von Mikroorganismen, die sich an der Sensoroberfläche angesiedelt haben, wird durch eine 280nm UV-LED angeregt. Bei einer Wellenlänge von 350nm werden Photonen emittiert, welche von ringförmig um die UV-LED angeordneten Glasfasern aufgesammelt werden. Über zwei optische Interferenzfilter gelangen die Photonen auf einen als Detektor fungierender Photoelektronenvervielfacher. In Simulationen des optischen Strahlengangs wurde die Anordnung der optischen Komponenten so optimiert, dass es möglich war, die Hintergrundfluoreszenz des Seewassers zu minimieren und die Biofilme selektiv an der Sensoroberfläche zu detektieren. Es konnte gezeigt werden, dass eine gewinkelte Anordnung der Glasfasern entscheidend zur Biofilm-Detektionseffizienz beiträgt. Unter Laborbedingungen wurde eine bakterielle Zelldichte von  $4 \times 10^3 - 10^7$  Zellen/cm<sup>2</sup> ermittelt, was einer prozentualen Bedeckung von 0,01-60% entspricht. Der Sensor verfügt über eine Datenerfassungselektronik und ist mit einem wasserdichten Gehäuse ausgestattet, was die quasi-kontinuierliche Messung der Biofilmsiedlung im Meer ermöglichte. In umfangreichen Feldversuchen in der Ostsee und im Pazifischem Ozean wurde die Biofilmsiedlung zu unterschiedlichen Jahreszeiten und Umweltbedingungen gemessen. Die initiale Ansiedlung von Bakterien erfolgt innerhalb der ersten Stunden. Die Messungen haben ergeben, dass die Siedlungsdynamik der Bakterien innerhalb der ersten 24 Stunden noch jahreszeitenunabhängig erfolgt. Bei Messungen über mehrere Wochen hingegen zeigte sich, dass jahreszeitliche

Einflüsse wie Temperatur und Nährstoffgehalt des Umgebungswassers signifikante Auswirkungen auf die Biofilmdynamik haben. Zudem konnte ein mathematisches Modell entwickelt werden, welches die Biofilmsiedlung und die verschiedenen Phasen der Entwicklung beschreibt. Anhand von epifluoreszenzmikroskopischen Aufnahmen von Referenzproben wurde dieses Modell verifiziert und bestätigt. Des Weiteren wurde eine tageszeitliche Rhythmik des Fluoreszenzsignals mit dem Biofilmsensor gemessen. Bei Untersuchungen der Siedlungsdynamik von Biofilmen auf künstlichen Substraten in der unmittelbaren Nähe der Makroalge *Fucus vesiculosus* konnte ein fördernder Einfluss auf die Wachstumsrate der Mikroorganismen festgestellt werden. Durch *F. vesiculosus* wurde weiterhin die bakterielle Biofilmgemeinschaft der Gruppen *Alphaproteobakterien* und *Cytophagabakterien* verändert. Daraufhin wurde die Bakteriengemeinschaft auf der Makroalge untersucht und es konnte eine hohe Dichte von *Alphaproteobakterien* festgestellt werden. Im Labor und unter Feldbedingungen wurden Oberflächenextrakte der Braunalge *Fucus vesiculosus* und deren Wirkung auf die bakterielle Ansiedlung untersucht. Es konnte gezeigt werden, dass diese einen quantitativen und qualitativen Einfluss auf die bakterielle Biofilmgemeinschaft haben. Innerhalb der komplexen Fraktionen der Algenmetaboliten konnten zwei Substanzen identifizieren werden, die für die Abwehr der *Gammaproteobakterien* verantwortlich sind.

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# Contents

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<b>Abstract</b>	<b>I</b>
<b>Zusammenfassung</b>	<b>III</b>
<b>Acknowledgment</b>	<b>V</b>
<b>1. General Introduction</b>	<b>1</b>
1.1. Bacterial Biofilms . . . . .	2
1.2. Influencing Factors and Consequences of Biofilm Formation . . . . .	3
1.3. State-of-the-Art Optical Biofilm Detection Methods . . . . .	5
1.3.1. Microscopic Techniques . . . . .	6
1.3.2. Spectroscopic Techniques . . . . .	6
1.4. Concept and Requirements for Biofilm Sensors in the Field . . . . .	10
1.5. Aims of the Project and Outline of this Thesis . . . . .	11
<b>2. Biofilm Sensor Development</b>	<b>14</b>
2.1. <b>Paper I:</b> Design and Field Application of a UV-LED Based Optical Fiber Biofilm Sensor . . . . .	15
2.1.1. Abstract . . . . .	16
2.2. Native Protein Fluorescence . . . . .	17
2.3. Fluorescence Light Source & Detector . . . . .	18
2.4. Optical Simulation Results . . . . .	21
2.5. Electronic Setup for the Biofilm Field Sensor . . . . .	28
2.6. Graphical User Interface . . . . .	30
2.7. <b>Paper II:</b> Field Sensor for <i>in situ</i> Detection of Marine Bacterial Biofilms . .	32
2.7.1. Introduction . . . . .	33
2.7.2. Biofilm sensor concept . . . . .	33
2.7.3. Field sensor layout . . . . .	34
2.7.4. Biofilm measurement sequence . . . . .	34
2.7.5. Detection range . . . . .	36

2.7.6. Field experiment . . . . .	36
2.7.7. Conclusion . . . . .	38
<b>3. Marine Biofilm Formation</b>	<b>40</b>
3.1. Marine Surface Colonization . . . . .	41
3.2. Mathematical Description of the Marine Biofilm Fouling Sequence . . . . .	44
3.3. <b>Paper III:</b> On Biofilm Formation - Dynamics in Natural Marine Environments	46
3.3.1. Abstract . . . . .	47
<b>4. Impact of Marine Macroalgae on Bacterial Biofilms</b>	<b>48</b>
4.1. Effects of macroalga on physical, biological and chemical water parameters .	49
4.2. <b>Paper IV:</b> Compounds associated with algal surfaces mediate epiphytic col- onization of the marine macroalga <i>Fucus vesiculosus</i> . . . . .	52
4.2.1. Abstract . . . . .	53
<b>5. Synthesis and Conclusion</b>	<b>54</b>
<b>6. Outlook</b>	<b>58</b>
<b>Bibliography</b>	<b>60</b>
<b>Eidesstattliche Erklärung</b>	<b>79</b>
<b>Appendix</b>	<b>80</b>
A. List of Abbreviations . . . . .	80
B. Published Patent Application . . . . .	83
C. List of Publications . . . . .	91



# General Introduction

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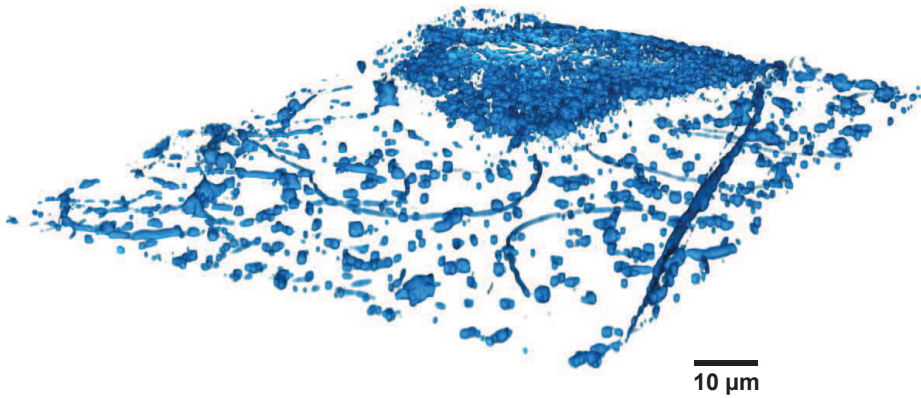
## 1.1. Bacterial Biofilms

NEARLY all submersed surfaces in aquatic environments become rapidly covered by microorganisms forming a biofilm. The majority of microbial activity in aquatic environments can be found at solid-liquid or air-liquid interfaces {Grossart, 2010}. Aquatic biofilms play an indispensable role in the food webs and biogeochemical pathways of carbon, nitrogen, hydrogen, sulphur, phosphorus and are the major component of the earth's biodiversity as reviewed by Azam and Malfatti {2007}. The marine biofilm formation and the interaction with its environment is described in more detail in Section 3.1. Bacterial biofilms are defined as a surface-associated bacterial community embedded within a self-produced matrix of extracellular polymer substances (EPS) {Costerton et al., 1999} as the net result of adsorption and desorption processes occurring simultaneously {Bryers and Characklis, 1982}.

In a first process on submerged surfaces, the adsorption of ions and molecules such as sugars, amino acids, proteins and fatty and humic acids starts immediately and a dynamic equilibrium is established within tens of minutes. Some of these substances, for instance sugars and amino acids, facilitate the settlement of bacteria and other microorganisms. Following this biochemical conditioning phase, free swimming pioneer bacteria cells settle on the surface. Investigations by Marshall et al. {1971} showed that the first stage of bacterial colonization of a surface is composed of a reversible adoption which is controlled by physical mechanisms such as Brownian motion, electrostatic interaction and Van-der-Waal forces {Fletcher and Loeb, 1979}. The second stage of biofilm formation corresponds to the irreversible attachment of microorganisms to the substrate forming a dispersed monolayer of bacteria cells. Some bacteria strains use either flagella or type IV pili to move along the surface until other bacteria are encountered and microcolonies, the basic structural and functional unit of the microbial biofilm, are formed {Costerton et al., 1995, Pratt and Kolter, 1998}. These microcolonies may be composed of cells of a single species or cells of several species which are clearly delineated by their EPS production. Moreover, it has been demonstrated that microcolonies are organized in a complex three-dimensional structure with an anastomosing network of water channels. The water channels penetrate to all levels of the biofilm and bring the bulk fluid phase amongst and even behind the bacterial microcolonies {Lawrence et al., 1991}. Thereby, the bacteria have to communicate with each other to develop a biofilm. They are capable of controlling the population density and to coordinate gene expression via quorum sensing signals. For instance the acylhomoserine lactone (AHL) system is essential for differentiated biofilms and affects several aspects of biofilm dynamics including heterogeneity, architecture, stress resistance, maintenance and sloughing {Bassler and Losick, 2006, Kjelleberg and Molin, 2002}. Finally, the matured "biofilm is less like a highly developed organism and more like a complex, highly differentiated, multicultural community much like our own city" {Watnick

and Kolter, 2000}.

According to Bryers and Characklis {1982} the dynamic multistep process of biofilm formation can be summarized by three main phases of development. The bacterial attachment phase includes the first cell approaches to the surface. It is followed by an exponential net accumulation phase with cellular reproduction and microbial production. Ultimately, a pseudo stationary phase commences while an ordered three-dimensional structure is developed and the rate of cell division equals the rate of cell death or dispersal. A 3-D reconstruction of a bacterial biofilm as given in this work on a surface is illustrated in (Figure 1.1). Blue areas alternate with white and denote single bacteria which form local colonies.



**Figure 1.1.:** 3-D reconstruction of a bacterial biofilm. The image was obtained by combining 50 horizontal optical image sections taken at  $0.2 \mu\text{m}$  intervals. The biofilm was stained with DAPI and visualized by applying epifluorescence microscopy in combination with the image analysis software ImageJ.

## 1.2. Influencing Factors and Consequences of Biofilm Formation

*Bacterial adhesion* - In general, the hydrodynamics of the liquid phase, environmental conditions such as temperature or pH, physiological and metabolic state of cells, substrate conditions, and production of EPS have been shown to affect levels of bacterial adhesion {Donlan, 2002, Rosenberg and Kjelleberg, 1986, Wahl, 1989, 1995}. An important factor in microbial colonization is the characteristics of the solid surface. Commonly, the microbial attachment rate increases with surface roughness because shear forces are diminished and the surface area is simply higher on rougher surfaces. Attachment strength of bacteria is mainly determined by the surface wettability and not necessarily by the surface tension. Becker {1998} showed that within a range of 20 and  $25 \text{ mNm}^{-1}$  bacterial adhesion is at its minimum. Moreover, it has been shown for surfaces with instance water content, for instant hydrogel polymers,

that attachment of bacteria decreases {Pringle and Fletcher, 1986}. Furthermore, the rate of microbial cell attachment is influenced by the properties of the bacterial species and their cell surface hydrophobicity as well as morphology. It has been demonstrated by Maki et al. {1990} that those polymer molecules on the cell surface used by bacteria for attachment are heterogeneous in their composition. The surface of bacteria is to some extent hydrophobic and mostly negatively charged {Fletcher and Loeb, 1979}. Therefore, strong adherence capacities toward hydrophobic substrates have been reported {Marshall et al., 1971} and settlement to hydrophilic surfaces is reduced. Dalton et al. {1994} reported that on hydrophobic surfaces, bacteria tend to form tightly packed biofilms consisting of single and paired cells in contrast to hydrophilic surfaces on which exhibited sparse chains with total lengths in excess of 100  $\mu\text{m}$  are formed.

*Occurrence* - The physical, biological and chemical environmental conditions exert major influences on biofilm formation dynamics. Overall, biofilms can tolerate various extreme hydrodynamic and environmental conditions of the aquatic environment. They are found in Morning Glory pool in Yellowstone Park as well as in acid mine drainage (at a pH 0) and in the abyssal areas of the oceans on black smokers. The latter habitat is characterized by extreme physical and chemical water conditions and strong hydrodynamics, thereby pressing the limits of survival of living organisms {Costerton, 2007, Davey and O'toole, 2000}.

*Biological function* - Living within a stable biofilm provides an ideal environment for metabolic cooperation within consortia of cells of different species such as the degradation and cycling of organic molecules and various nutrients (e.g., nitrogen, sulfur, and carbon), exchange of extrachromosomal deoxyribonucleic acid (DNA), and quorum sensing {Donlan, 2002}. Mature biofilms, can tolerate antimicrobial substances in 10-1000 times {Lewis, 2001} higher concentrations as planktonic microorganisms and are even better resistant to phagocytosis. In addition to the advantage of resistance to environmental changes, the biofilm may benefit from a number of properties of a communal existence including division of the metabolic burden, gene transfer, and cooperative, unselfish behavior {Jefferson, 2004}. Biofilm formation may therefore be a survival mechanism for bacteria and other microbes and provides a more favorable environment {Watnick and Kolter, 1999}. In contrast to these favorable characteristics of biofilm, a potentially adverse consequence of bacterial growth in complex structure is that some cells in the interior of a microcolony will be overgrown. Then they get further from a bulk liquid interface, hence away from essential sources of energy or nutrients. Moreover, waste products as well as toxins can accumulate to dangerous levels within such zones. This often leads to cell death in the center of the microcolony, increasing the probability of collapse of the entire cell cluster {Fagerlind et al., 2011}. Furthermore, grazing pressure and moisture content can life-threaten an entire biofilm community. Therefore bacterial biofilms have the opportunity to convert back to the planktonic life. This life cycle of biofilm dispersal seems to

be activated by nitric oxide and subsequent quorum sensing signals to alter gene expression and metabolic activity. Now, bacteria of a microcolony develop dispersal variances which are able to subsequently discover new habitats {Steinberg et al., 2011}.

*Significance in daily life* - Biofilms play an important role in our daily life as they form on industrially engineered aquatic systems causing processes like corrosion and fouling {Simões et al., 2010}. Microbial colonization can reduce heat or mass transfer on heat exchangers, condensers and membranes {Flemming, 2002}. In a medical context, bacterial biofilms cause major problems in dental hygiene, infection diseases and infections related to medical implants and develop on all types of devices, heart valves, contact lenses, urinary, endotracheal, intravenous, and other types of catheters {Donlan, 2001}. The National Institute of Health (USA) estimated that more than 80% of the bacterial infections are biofilm related and the mortality of the patients associated with biofilms is substantial {Wolcott and Ehrlich, 2008}. Device-related infections constitute a major cause of bacterial infections in hospitalized patients {Costerton et al., 1999, Donlan, 2002, Monroe, 2007}. On the other hand microbial biofilms help to remove contaminants from the soil or ground water and in trickling filters. In natural habitats such as rivers, lakes, and coastal areas the major metabolic and nutrients cycling activities occur in microbial biofilms {Azam and Malfatti, 2007, Davey and O'toole, 2000}.

### 1.3. State-of-the-Art Optical Biofilm Detection Methods

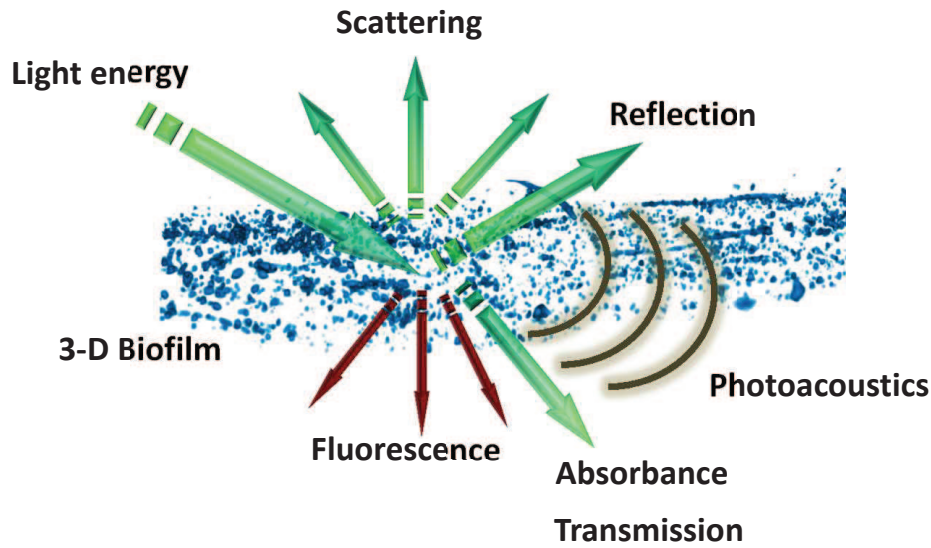
The key to characterize the formation of biofilms is a continuous monitoring of quantitative and qualitative composition of the film. Along those lines, several monitoring techniques based on biofilm induced changes of electrical conductivity or capacity, heat transfer, friction, intensity of sound and light have been developed. Generally, all online biofilm monitoring techniques are based on a signal obtained from the biofilm and can be divided into direct measurements for estimating biomass and cell density and indirect examinations of metabolic activity and products like liquids or gases. An overview of sensor strategies available to date has been summarized by several authors {Denkhaus et al., 2006, Dickert et al., 2003, Flemming, 2003, Janknecht and Melo, 2003, Mehrvar et al., 2000, Nivens et al., 1995}. The scientific area of biofilm sensors is a very broad and complex field and comprises many different and independent research areas. Consequently this section cannot give a comprehensive overview on the complete research of biofilm sensors, but rather concentrates on optical sensing techniques. The scope of this chapter is to review key achievements of optical biofilm sensors with respect to their high potential for field applications. A review of optical and spectroscopic techniques for examination of biofilms and their detailed descriptions including methods of microscopy has been published by Wolf et al. {2002}.

### 1.3.1. Microscopic Techniques

Microscopy enables the detection of biofilm microorganisms down to a single cell level. The first observation of bacteria in a dental biofilm was investigated by a single lens microscope by A. van Leeuwenhoek in 1684. Since this time, microscopy in combination with computerized image analysis is of paramount importance and a basic tool for biofilm research. For investigating the community composition and quantification, light, episcopic differential interference contrast (EDIC) {Rogers and Keevil, 1992}, epifluorescence and confocal laser scanning microscopy (CLSM) in combination with fluorochrome staining of the biofilm are commonly applied. Fluorescent dyes commonly used in biofilm detection are acrydine orange (AO) {McCoy and Olson, 1985}, 4,6-diamidino-2-phenylindole (DAPI) {Sieracki et al., 1985} and Hoechst 33258 {McCoy and Olson, 1985} targeting the DNA of microorganisms. Cyanine dyes like Cy3 or Cy5 in combination with an oligonucleotide ribosomal ribonucleic acid (rRNA) specific probe are used for staining specific bacterial biofilm community members. A review on fluorochrome staining techniques was published by McFeters et al. {1995}. Methods of electron microscopy such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been of special importance in elucidating biofilm structure {Richards and Turner, 1984}. Scanning transmission x-ray microscopy (STXM) is applied for studying initial steps of bacterial colonization and of visualizing diverse groups of molecular features and structures of surfaces by detection of chemical elements and their binding states {Gilbert et al., 1999}. Another microscopy method recently employed in biofilm monitoring is atomic force microscopy (AFM), able to elicit biofilm surface topography at resolutions down to the atomic level {Surman et al., 1996}. These microscopy techniques provide an excellent sensitivity, however they are mostly applicable in the laboratory and are less suitable for monitoring biofilm formation in the field.

### 1.3.2. Spectroscopic Techniques

An increasing number of biofilm monitoring systems is based on the detection of ultraviolet, visible and infrared light. Five main interactions between electromagnetic radiation and biofilm matter are utilized to examining biofilm formation dynamics: absorption/transmission, reflection, scattering, fluorescence, and photoacoustics as schematically shown in Figure 1.2. Moreover, bioluminescence has been applied for investigating biofilms. The following sections illustrate the optical methods for investigating biofilms on the basis of representative publications.



**Figure 1.2.:** Main interactions between light and biofilms which are applied for exploring microbial surface colonization.

### **Bioluminescence**

Bioluminescence occurs spontaneously in the biofilm when for instance luciferin is oxidized in the presence of adenosine triphosphate (ATP) and the enzyme luciferase {Lazarova et al., 1995}. This natural light generation process is limited to relatively few organisms and depends on external conditions such as salt, ammonium and nitrate concentrations {Rice et al., 1995}. Bioluminescence has been used to determine biomass and may be applied as marker for cellular stress or toxicity in biofilms {Angell et al., 1993} under laboratory conditions.

### **Reflection and scattering**

Light reflection and scattering methods for biofilm detection have been developed based on differential turbidity measurements (DTM) {Flemming et al., 1998}. These fiber optic sensors (FOS) are based on the determination of the local concentration of light scattering particles deposited on an optical fiber tip and have been applied in water pipes to detect biofilm formation {Tamachkiorow and Flemming, 2003}. Beyenal et al. {2000} built an optical microsensor based on commercially available fiber optic telecommunications components. Two laser light sources at 1320 and 670 nm were applied to measure spatially resolved profiles of backscattered light in a biofilm. The observed response signal agrees well with conventional local effective diffusivity microelectrode measurements. The drawbacks of those systems are that they are not only selective to microbial deposits, but also incorporate backscattering from abiotic particles {Flemming et al., 1998}. Optical coherence tomography (OCT) is a three-



dimensional interferometric image acquisition technique with micrometer scale resolution and typically employing near infrared (NIR) light. The backscattered light signal reflects the relative optical density distribution in the biofilm. A lateral and axial resolution in the range of 10  $\mu\text{m}$  has been achieved and transient processes with a temporal resolution in a second to minute scale have been recorded by Haisch and Niessner {2007}. OCT enables the visualization of the complete heterotrophic biofilm structure including the substrate, the pores, and connected structures simultaneously {Neu et al., 2010}.

The inelastic scattering of a photon from an atom or molecule, called Raman effect, can also be used for examining biofilms. Surface-enhanced Raman scattering (SERS) has been applied to detect biomolecules at very low concentration levels and their relative abundance in a multispecies biofilm matrix grown in a funnel reactor {Ivleva et al., 2008}. Moreover, it has been shown that Raman spectroscopy is capable of systematic and rapid identification of clinically important bacterial biofilms and is able to obtain a "fingerprint" of a bacterial colony to distinguish different bacteria strains {Samek et al., 2010}.

### Absorption

Conventional absorption spectroscopy with fiber optic spectrometers is utilized for screening populations of phototrophic bacteria colonies and benthic cyanobacteria mats. These pigmented microorganisms exhibit several dominant absorption maxima due to chlorophyll *a* at 450 and 680 nm, carotenoids around 450-500 nm, phycocyanin at 620 nm, and bacteriochlorophyll *a* around 800-900 nm {Wiggli et al., 1996}. A simple monitoring system for detecting biofilm accumulation, growth and removal on a transparent surface by using a NIR emitter at a wavelength of 950 nm has been demonstrated by Bartlett et al. {2000} and Tingham and Bott {2003}. NIR emitter and detector were mounted on the outside of glass tubes and at the inner walls a biofilm was growing. The resulting signal could be correlated with biofilm thickness.

A surface sensitive infrared (IR) technique for biofilm examination employing attenuated total reflection (ATR) in conjunction with Fourier transform infrared (FTIR) spectroscopy has been described by Nivens et al. {1993}. The biofilms are grown and sampled on an internal reflection element used as substrate. A beam of IR-radiation penetrates through this element and upon each total internal reflection the evanescent field of the reflected light interacts with the biofilm. FTIR-ATR spectroscopy is employed to evaluate changes in metabolic activity, quantity and kinetics of thin biofilms as the penetration depth of evanescent waves is only a few microns {Keirsse et al., 2006, Nivens et al., 1993, Schmitt and Flemming, 1998}. Similar to ATR spectroscopy surface plasmon resonance (SPR) relies on the excitation of surface plasmons when light is reflected from a thin metal layer deposited on



a prism. Typically, the excitation of the surface plasmon reduces the reflected light intensity at a well defined incident angle of the analysis light beam. The exact angle sensitivity depends on the interfacial characteristics {Subramanian et al., 2006}. Interaction of the biofilm with the surface induces a change in the refractive index of the interface and causes a shift in the angle of resonance, which is proportional to the mass of biofilm bound to the surface {Leitz et al., 2002, Zourob et al., 2005}.

Moreover, biofilms have been investigated by a combination of light absorption and sound detection. So-called pulsed photoacoustic spectroscopy (PPS) is based on the absorption of electromagnetic radiation inside a biofilm and its conversion into heat. Due to the pulsed thermal expansion of the biofilm, a pressure wave is generated which can be detected by microphones or other piezoelectric transducers. The sound intensity of the detected signal is proportional to the optical absorption coefficient of the biofilm {Schmid et al., 2003} which in turn can be correlated to the thickness of the film.

## Fluorescence

The phenomenon of photoluminescence is the spontaneous re-emission of absorbed light from any substrate, and is classified as fluorescence and phosphorescence. The absorption of light energy promotes an electron to an electronically excited state. Before the excited electron returns to the ground state some energy is lost in transitions between vibrational energy levels (vibrational relaxation), and a photon at a longer wavelength is emitted (Stokes shift). Transitions between electronic states with the same spin multiplicity (e.g., singlet-singlet transitions) are called fluorescence, otherwise phosphorescence (e.g., triplet-singlet transitions). The ratio of the number of photons emitted to the number absorbed is defined by the quantum yield {Lakowicz, 1999}. Fluorescence that typically occurs from aromatic molecules is known as intrinsic or natural fluorescence. A list of the spectral characteristics of biological molecules in microorganisms that exhibit intrinsic fluorescence, along with their excitation and emission maxima can be found in Table 1.1. Biofilm microorganisms contain a variety of intracellular biomolecules that have specific excitation and emission wavelength spectra characterizing their intrinsic fluorescence. Monitoring of biomolecules based on intracellular fluorophores, for instance tryptophan, flavin adenine dinucleotide (FAD), the reduced form of nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) and pyridoxine, has long been known {Arrage et al., 1995, Determann, 1998, Sohn et al., 2009, Tartakovsky, 1996}. A fluorescence spectrum of a biofilm can reveal qualitative structural, quantitative and physiological information of microorganisms {Li and Humphrey, 1991, Mittelman et al., 1993}. Two-dimensional fluorometry monitors a range of excitation and emission wavelengths simultaneously, resulting in a three-dimensional fluorescence map. For example, 2D-fluorometry was used for non-invasive, *in situ* monitoring of a membrane-attached multispecies biofilm

reactor. The fluorescence spectra were recorded in a range of 280 to 510 nm for excitation and fluorescence emission was detected from 305 to 545 nm in synchronous scanning mode {Wolf et al., 2001}.

**Table 1.1.:** Typical intrinsic fluorophores with their excitation maximum ( $\lambda_{\text{ex}}$ ) and emission ( $\lambda_{\text{em}}$ ) characteristics.

Intrinsic fluorophore	$\lambda_{\text{ex}}$ (nm)	$\lambda_{\text{em}}$ (nm)	Reference
DNA, RNA	260	320	{Pisarevskii et al., 1966}
Aromatic amino acids			
Tryptophan	280	350	{Bliss, 1979}
Tyrosine	275	300	{Determann, 1998}
Phenylalanine	260	280	{Ammor, 2007}
Enzymes, coenzymes, vitamins			
NADH	290	440	{Leblanc and Dufour, 2002}
NADPH	336	464	{Li and Humphrey, 1991}
FAD	450	535	{Kurilcik et al., 2006}
Pyridoxal phosphate	280	400	{Möller and Denicola, 2002}
Pterins	350	450	{Alimova et al., 2003}
Riboflavin	450	525	{Tartakovsky, 1996}
Pigments			
Chlorophyll <i>a</i>	450	680	{Kühl, 2005}
Phycoerythrin	565	573	{Neu et al., 2004}
Fucoxanthin	440	675	{Gundermann and Büchel, 2012}
Bacteriochlorophyll <i>a</i>	360	790	{Koblížek et al., 2005}

## 1.4. Concept and Requirements for Biofilm Sensors in the Field

After transferring a natural microbial community, to culture them under laboratory conditions, a "bottle effect" has been observed {Heukelekian and Heller, 1940, Zobell, 1943}. Laboratory cultures of bacterial strains have indicated a loss of a fraction of their diversity of genes whereby bacterial growth and activity were substantially enhanced {Fux et al., 2005}. This is just one reason why, it is of substantial importance to develop compact and robust field

sensors that allow *in situ* measurements of biofilm development. The availability of a biofilm field sensor opens up entirely new research opportunities in microbial ecology, biophysics and marine technology. Owing to technical limitation or complexity of the instruments, the majority of biofilm detection methods are merely applicable in the laboratory rather than in field studies or other harsh environments {Janknecht and Melo, 2003}. Optically continuous monitoring of biofilm formation dynamics in the field has to meet challenges which differ from highly sophisticated laboratory instrumentation as described in Section 1.3. First of all, it has to be considered that a natural biofilm is almost invariably a multispecies microbial community and can mature to three-dimensional, highly complex cell clusters {Stoodley et al., 2001}. Optical methods based on fluorescence have been proven useful for quantitative detection of biomass and cellular activity {Angell et al., 1993, Wolf et al., 2001} and yield signals that correlate with cell number and bacteria growth {Ji et al., 2004}. In particular, the high sensitivity, fast response time, and the capability of biofilm detection *in situ* without sample contact make fluorescence methods attractive for monitoring of biomolecules using intracellular fluorophores. Moreover, the sensor concept should allow for non-destructive detection in order to facilitate online measurements, selective detection of the biofilm to distinguish between organic and inorganic material on the surface, background suppression of signal attributable to organic material in the overlaying bulk water, and sufficient penetration depth accounting for the biofilm's three-dimensional structure. As complex cell clusters and patchy microcolonies of several hundred micrometer in diameter {Dalton et al., 1996, Stoodley et al., 2001} are formed by the biofilm, the detection area has to be larger than  $0.5 \text{ cm}^2$  to achieve a homogenous overview of the biofilm constituents. A detection range is required which gives the opportunity to monitor microbial colonization from initial attachment of bacteria cells to an established and mature biofilm under field conditions. This process may take days to weeks calling for an autonomous operating sensor with the possibility of a sampling frequency in the range of a few minutes to detect short-term variations of microbial settlement. Furthermore, crucial issues of an environmental sensor are its robustness, portability, low power consumption and minimal assembly of all optical and electronic parts.

## 1.5. Aims of the Project and Outline of this Thesis

Within the scope of this thesis, a number of results were obtained that contribute fundamentally to the current knowledge about marine biofilm formation dynamics. At the beginning of this project, continuous and *in situ* detection of biofilm establishment in the marine habitat with the required fine temporal and spatial resolution was not feasible. Therefore, one of the main project tasks was the development of an optical field sensor which employs fluorescence intensity of the amino acid tryptophan as indicator for cell number and surface coverage. This

sensor should be used to explore fundamental biological questions of the formation dynamics of marine biofilms. This work combines applied and interdisciplinary aspects of different scientific fields such as biophotonics, marine microbial ecology and chemical ecology.

A comprehensive experimental program was initiated to systematically investigate *in situ* marine biofilm formation under different physical, biological, chemical environmental conditions such as temperature, dissolved oxygen concentration, and pH. Interactions between macroalgae and biofilms mediated by released chemical and biochemical substances, has been studied as well. It was possible to explore the potential sensitivity of the biofilm community structure towards seasonal variations and locations as well as macroalgae surface extracts.

Chapter 2 describes the development of an optical fiber biofilm sensor based on detecting intrinsic tryptophan fluorescence of microorganisms. Promising sensor geometries have been evaluated in order to identify and visualize the maximal fluorescence collection performance by using FORTRAN software based on ray-tracing methodology. As a result, the sensor head principle allows for large-area illumination and detection and thus ensures the required averaging over the patchy structured biofilms. Moreover, the detection range has been estimated and practically verified under laboratory conditions with two marine bacteria strains. Two prototype sensor configurations were assembled and tested. Successful field experiments in the Baltic Sea were accomplished and provided for the first time a quasi-continuous observation of biofilm formation dynamics in a marine habitat.

Chapter 3 of this thesis concentrates on field experiments by exposing the developed sensor to different marine environments. It turned out that a minimum temporal resolution of a few minutes is useful to investigate the attachment dynamics of biofilm forming bacteria continuously. In parallel to the investigation of bacterial biofilm dynamics, the composition of the primary bacterial community of the establishing biofilm was identified by using fluorescence *in situ* hybridization (FISH) in combination with epifluorescence microscopy. In parallel, to get a better understanding of the main drivers of biofilm formation dynamics, the characterization of the microhabitat in terms of physical, biological and chemical water parameters is of basic interest. Concluding with studies of settling dynamics of fouling microorganisms such as bacteria, diatoms, alga spores in marine environments, it was possible to develop a basic model for biofilm formation dynamics.

At present, very little is known about the *in situ* interaction of marine biofilm dynamics in presence of the bladder wrack *Fucus vesiculosus* that exudes pro- and antifouling compounds. In Section 3.3, the discovery of a stimulatory effect on bacterial settlement when *F. vesiculosus* was in close proximity will be described. Based on this result, Chapter 4.1 addresses the question which chemical pro- and antifouling compounds of the algae do affect the bacterial settlement and community composition in the marine habitat. Thereby, compounds on algal

thalli were separated from the surface by extraction with organic solvents. Purified extract fractions were exposed to bacterial biofilms in the laboratory and in the marine environment followed by bacterial abundance and community analysis.

# Biofilm Sensor Development

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# Paper I

## 2.1. Design and Field Application of a UV-LED Based Optical Fiber Biofilm Sensor

M. Fischer<sup>a</sup>, M. Wahl<sup>a</sup> and G. Friedrichs<sup>b,\*</sup>

<sup>a</sup> GEOMAR | Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

<sup>b</sup> Institute of Physical Chemistry, Christian-Albrechts-University Kiel, Kiel, Germany

\* To whom correspondence should be addressed.

E-mail address: friedrichs@phc.uni-kiel.de (G. Friedrichs).

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Contribution of M. Fischer

- Development and assembly of biofilm sensor
- Numerical simulations of sensor head geometry and performance
- Experimental work and data evaluation
- Preparation of the raw version of the paper

Contribution of Co-authors

- Supervision of the experimental work, joint design of sensor concept
- FORTRAN programming
- Completion of final paper

### 2.1.1. Abstract

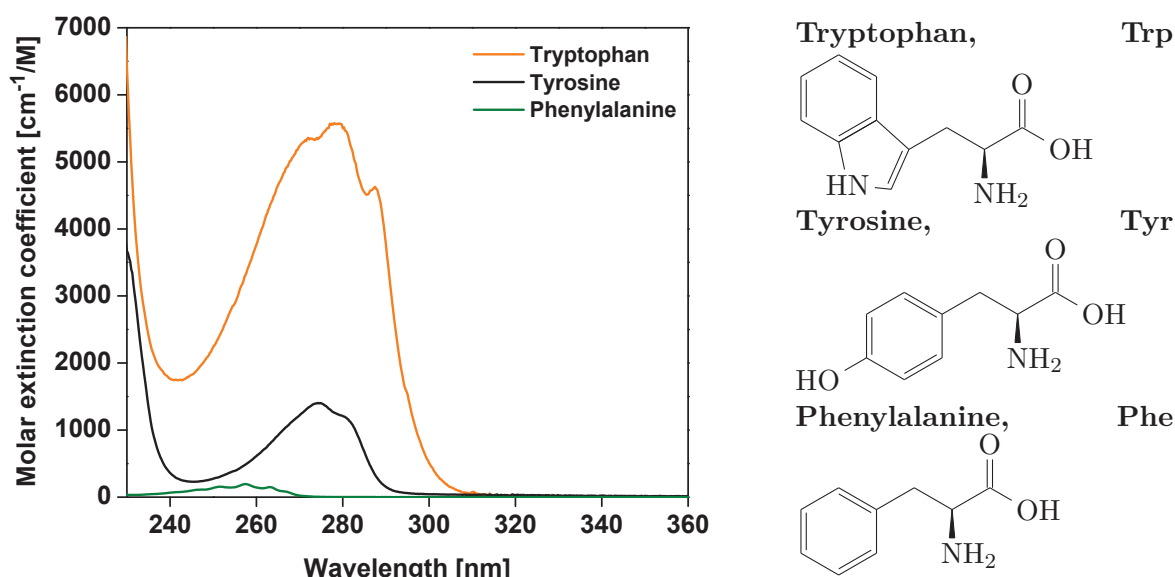
**D**ETECTING changes in the formation dynamics of biofilms stemming from bacteria and unicellular microorganisms in their natural environment is of prime interest for biological, ecological as well as anti-fouling technology research. We developed a robust optical fiber-based biofilm sensor ready to be applied in natural aquatic environments for on-line, *in situ* and non-destructive monitoring of large-area biofilms. The device is based on the detection of the natural fluorescence of microorganisms constituting the biofilm. Basically, the intrinsic fluorescence of the amino acid tryptophan is excited at a wavelength of  $\lambda=280$  nm and detected at  $\lambda=350$  nm utilising a numerically optimized sensor head equipped with a UV-LED light source and optical fiber bundles for efficient fluorescence light collection. Calibration was carried out with tryptophan solutions and two characteristic marine bacteria strains revealing linear signal response, satisfactory background suppression, wide dynamic range, and an experimental detection limit of  $4 \times 10^3$  cells/cm<sup>2</sup>. Successful field experiments in the Baltic Sea accomplished over a period of twenty-one days provided for the first time continuous observation of biofilm formation dynamics in a natural habitat. Starting from the first adhering bacteria, the measurement yielded the characteristic three phases of biofilm formation up to a fully developed biofilm. The sensor system holds potential for applications in aquatic sciences including deep sea research and, after further miniaturisation, in the industrial and biomedical field.



## 2.2. Native Protein Fluorescence

Intrinsic protein fluorescence in the ultraviolet (UV) range originates mainly from the three aromatic amino acids tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp) {Lakowicz, 1999}. Their specific absorption spectra and chemical structures are shown in Figure 2.1. Due to a very low quantum yield of Phe and common quenching mechanisms such as resonance energy transfer (RET) from Tyr to Trp, the native fluorescence in proteins is dominated by tryptophan.

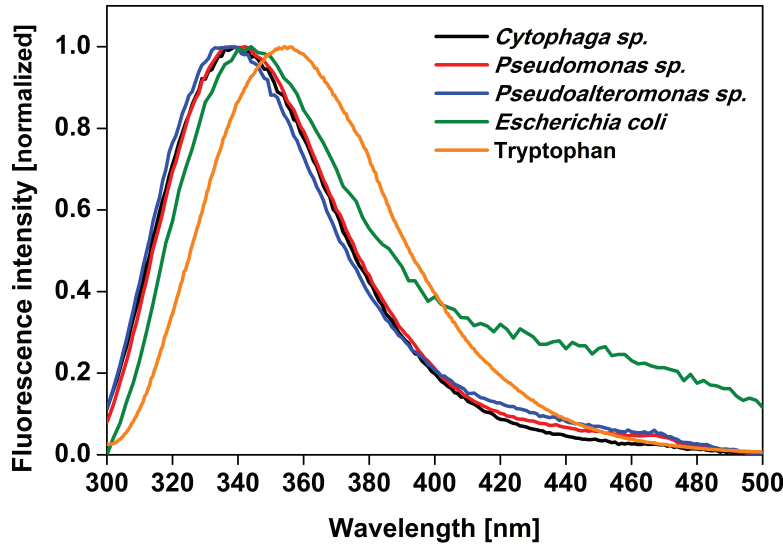
L-tryptophan [(2S)-2-amino-3-(1H-indol-3-yl)propanoic acid] is one of the essential 22 amino acids with the molecular formula  $C_{11}H_{12}N_2O_2$  and is utilized by organisms to synthesize the proteins. The indole groups of tryptophan can be selectively measured by optical excitation at  $\lambda = 280$  nm and fluorescence signal is collected at  $350 \text{ nm} < \lambda < 450 \text{ nm}$  with its peak at  $\lambda = 350$  nm.



**Figure 2.1.:** Molar extinction coefficient and chemical structures of the three aromatic amino acids dissolved in water and 0.1 M phosphate buffer at pH 7. The spectral data were downloaded from <http://omlc.orgi.edu/spectra/PhotochemCAD/index.html>.

Proteins in bacteria naturally emit fluorescent light in the wavelength range of  $300 \text{ nm} < \lambda < 400 \text{ nm}$  with an emission maximum at  $\lambda = 340$  nm following excitation at  $\lambda = 280$  nm of their tryptophan residues. However, the emission spectrum of tryptophan is sensitive to solvent polarity. In fact, a variation of the emission maximum can be seen in response to protein conformational transitions, subunit association, ligand binding, or denaturation, all of which can affect the local environment surrounding the indole ring. If

the tryptophan residue is buried in a hydrophobic environment within a protein occurring in bacteria, the emission may be blue-shifted as demonstrated in Figure 2.2 compared to pure tryptophan {Vivian and Callis, 2001}. Additionally, tryptophan that is bound to a protein displays a higher quantum yield than unbound tryptophan.



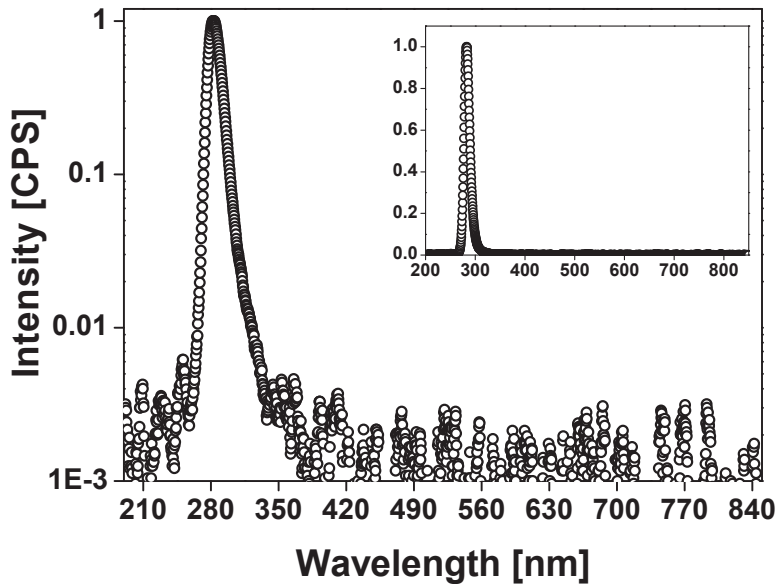
**Figure 2.2.:** Measured fluorescence emission spectra of three bacteria strains diluted in seawater and excited by a wavelength of  $\lambda = 280$  nm. Their characteristic fluorescence maxima are blue-shifted towards  $\lambda = 340$  nm due to naturally bound tryptophan {Ammor, 2007}. The seawater blank has been subtracted to remove the Raman scattering peak of water at  $\lambda = 309$  nm.

## 2.3. Fluorescence Light Source & Detector

*Light source* - The most commonly used light sources in fluorescence spectroscopy are high pressure xenon and mercury lamps which provide a relatively continuous light output covering a spectral range from  $250 \text{ nm} < \lambda < 700 \text{ nm}$ . Prior exposure of the sample, the excitation beam of these light sources has to be focused and spectrally separated by monochromators and/or optical filters. The power consumption is about 25 A at 20 V for a typical lamp with 500 W and they require high voltage pulses of 20 to 40 kV during starting phase. In contrast to these lamps, laser diodes emit quasi-monochromatic, collimated light. However, they are only available at wavelengths from  $385 \text{ nm} < \lambda < 1.5 \mu\text{m}$  {Lakowicz, 1999}. Alternatively, to obtain an optical output in the UV range ( $< 400 \text{ nm}$ ), technically more advanced laser systems would be applied. For example Storrie-Lombardi et al. {2001} demonstrated the use of the harmonic content of a mode-locked deep-UV He-Ag laser as a field deployable laser source. However, UV laser systems are not yet optimally suited for sensor applications due

to their large weight, size, high energy consumption and price.

At the outset of this thesis, a novel UV light source became available. Originally presented by McGuinness et al. {2005}, a solid-state aluminium gallium nitride (AlGaN) light emitting diode (LED) became commercially deliverable. A characteristic emission spectrum of a 280 nm LED operated at  $I = 20$  mA is shown in Figure 2.3 taken with a forward voltage as low as  $U = 5.7$  V. With an optical output power of about  $P = 600 \mu\text{W}$ , the spectrum shows a narrow spectral bandwidth of 14 nm at full width at half maximum (FWHM) and is free from additional emission bands up to a wavelength of 840 nm. Nevertheless, owing to the narrow separation between fluorescence excitation at  $\lambda = 280$  nm and tryptophan emission fluorescence detection window between 300 to 400 nm, separation of fluorescence light has to rely on further narrowing of the excitation spectrum by using appropriate excitation and emission filters. The LED is supplied in a hermetically sealed TO-18 housing without a need of external cooling.

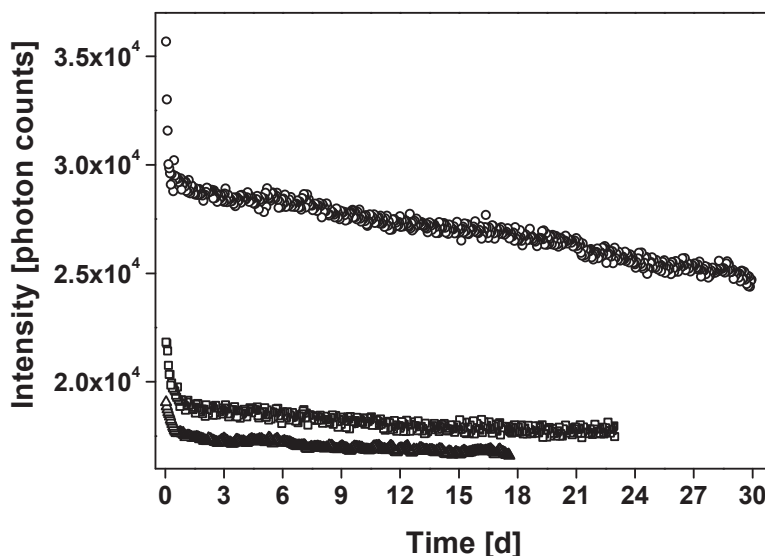


**Figure 2.3.:** Logarithmic and linear (inset) plot of the normalized electroluminescence spectrum of a UV-LED with a peak wavelength  $\lambda = 280$  nm.

Key advantages of applying UV-LEDs in field applications compared to other light sources used for fluorescence excitation are the low power consumption of 150 mW, the relatively narrow spectral bandwidth and low cost. Moreover, LEDs can be switched on and off instantaneously within several milliseconds and the output light can be easily amplitude modulated or pulsed.

Disadvantageous of the state-of-the-art UV-LED are aging processes. The photon emission

slowly decreases over time and the operating life time is limited to approximately one hundred hours. In order to demonstrate the stability of the UV light source, the output intensity of the UV-LED has been monitored over periods of several weeks (Figure 2.4). Here, the LED has been switched on for 10 ms once an hour and the emission intensity at 280 nm has been monitored with a single photon counting detector using a tabletop laboratory setup.



**Figure 2.4.:** Three intensity patterns of a used UV-LED over 30 (triangles), 23 (squares) and 17 (dots) days measured hourly.

The data clearly show a linear decay of the emitted light intensity over three time periods. Consequently, for high-precision or long-term measurements, the UV emission output of the LED needs to be monitored with a reference channel or investigated by regularly measuring a dilution series of tryptophan as calibration standard.

*Detector* - A photodetector measures the number of impinging photons per time interval by conversion of the absorbed energy into an electrical signal based on the internal and/or external photoelectric effect. Generally, three classes of photodetectors are available for sensitive fluorescence measurements which are photomultiplier tubes (PMTs), avalanche photodiodes and common photodiodes. The most sensitive signal response to individual photons applied in fluorescence spectroscopy is met by photomultiplier tubes. For measurements of intrinsic tryptophan fluorescence bi-alkali PMTs have been widely applied as adequate detector for single photon counting in the spectral range of 300 - 500 nm {Lakowicz, 1999}. Therefore, a PMT (Hamamatsu Photonics, Japan, H9319-01) has been applied for sensitive fluorescence light detection of the biofilm. The main optical characteristics of the detector are summarized in Table 2.1. This detector includes a 22-mm diameter head-on photomultiplier tube, a

photon counting circuit, a high-voltage power supply circuit, counter and a microprocessor.

**Table 2.1.:** Optical characteristics of the applied PMT detector for measuring fluorescence intensity of the biofilm.

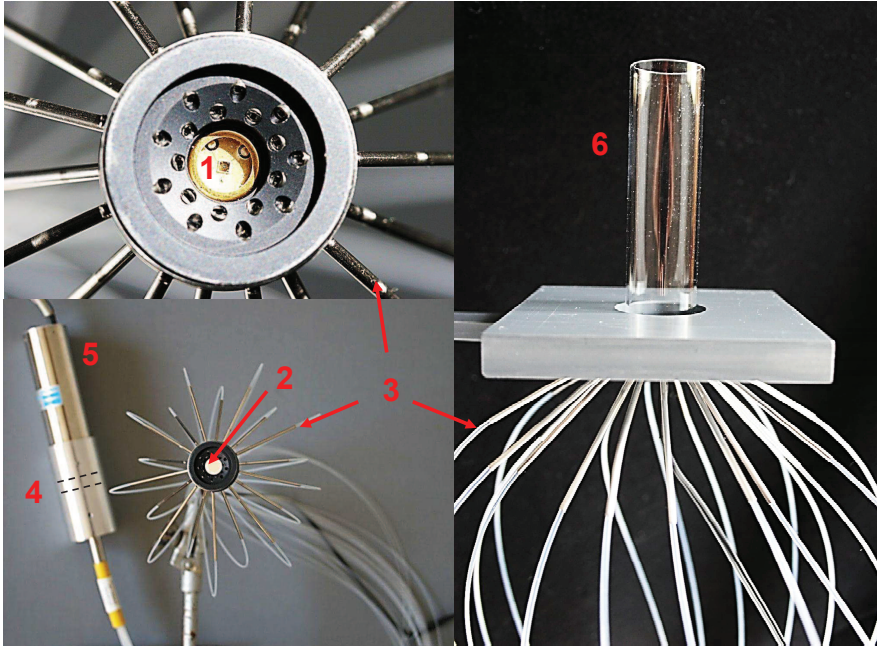
Parameter	Value	Unit
Peak sensitivity wavelength	420	nm
Count sensitivity at $\lambda = 420$ nm	$4.1 \times 10^5$	$\text{s}^{-1} \cdot \text{pW}^{-1}$
Count sensitivity at $\lambda = 350$ nm	$3.9 \times 10^5$	$\text{s}^{-1} \cdot \text{pW}^{-1}$
Count linearity	$2.0 \times 10^7$	$\text{s}^{-1}$
Dark counts at $U = 1050$ V	150	$\text{s}^{-1}$

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## 2.4. Optical Simulation Results

There are several important reasons to simulate and optimize the optical excitation and collection performance of the biofilm sensor head. First, a uniform  $0.5 \text{ cm}^2$  large biofilm sensing area is desired to allow for adequate averaging over its heterogeneous and patchy distribution due to the stochastic nature of biofilm development. Secondly, the signal should mainly arise from a detection volume directly from in front of the substrate to ensure the required specificity to suppress background fluorescence collection from the bulk water as much as possible. Along those lines, the number, the position, and inclination angels of the detection fibers were subject to optimization as they have a direct influence on the biofilm collection efficiency. Thirdly, the thickness of the protection window with removable biofilm settling substrates is determined by the maximal operating depth of the sensor. Other design parameters that influence the performance of the sensor head are: the size and angular emission pattern of the UV-LED, and the numerical apertures and diameters of the optical fibers. The latter determines the mechanical flexibility and bending radius of the fibers.

Based on a first table top prototype as illustrated in Figure 2.5, additional optimizations of the optical geometry were performed by simulating the specific parameters of the sensor and calculating the BCE. For simulation of the optical geometry, the spatial dimensions are discretized into a grid area containing equidistant rectangular elements. A set of test simulations showed that the results do not change significantly if the integration step width of the simulation grid was decreased below  $0.2 \text{ mm}$ . Therefore,  $0.2 \text{ mm}$  was chosen as the integration step width to precisely compute and evaluate a given geometry of the sensor head. As indicator for optimally designed probe geometry the program calculated the BCE as given



**Figure 2.5.:** The photographs show the first prototype setup of the sensor with 19 optical fibers (3) surrounding a UV-LED (1) with excitation filter (2) and the PMT detector (5) with indicated location of emission filters (4) as well as a calibration cuvette (6).

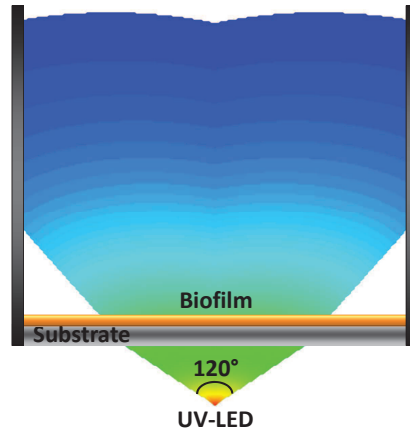
in equation 2.1. of the paper (Section 2.1.1). In this way, the parameter combination with the highest BCE value is regarded as optimal probe configuration.

The angular emission characteristics of the UV-LED, the size of the detection area and the detection efficiency field were simulated by level-by-level integration over all grid points based on ray-tracing methodology. The detection area from which 95% of the total biofilm fluorescence results from, is calculated from the detection efficiency pattern at biofilm level. A detailed description of the model parameters determining the sensor geometry is given in Section 2.1.1 of paper 1. A schematic cross-sectional view of the sensor head is illustrated in Figure 2.2. Typical parameters used in the simulation runs are summarized in Table 2.2.

The numerical simulations are performed as follows. In a first routine, a 3-D excitation light field of the UV-LED was numerically set up with the angular intensity distribution and the irradiance spatial pattern of the UV-LED as it is shown in Figure 2.6. The photon flux of the UV-LED decreases quadratically with the normal distance above the light source and refraction at the substrate window and optical excitation filter occurs.

**Table 2.2.:** Summary of typical model parameters (see also Figure 2.1.1).

Parameter	Description	Value	Dimension
$a$	Distance between biofilm and LED	11	mm
$b$	Thickness of the optical window	3	mm
$c, d$	Inclination angles	30, 45	$^{\circ}$
$e$	Radius of the side limit	10	mm
$f$	Half radiation angle of LED	60	$^{\circ}$
$g$	Half acceptance angle of the fibers	30	$^{\circ}$
$h$	Thickness of the fiber bundles	1.5	mm



**Figure 2.6.:** Simulated radiation pattern of the UV-LED which describes the relative intensity distribution in any direction from the light source. Red color corresponds to high and blue color to low intensity.

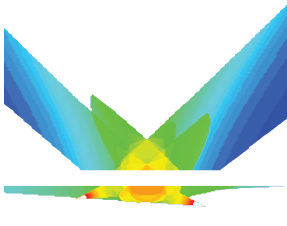
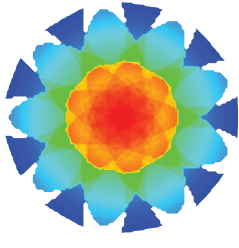
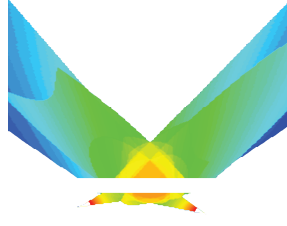
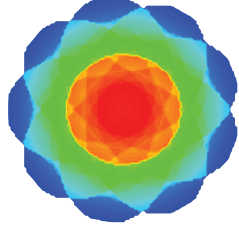

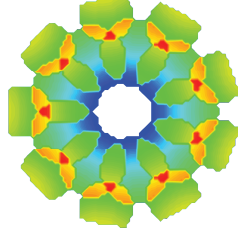
A second routine of the program simulates the biofilm which spherically emits photons at any point above the substrate. The resulting collection light field also takes into account Fresnel reflection and refraction occurring at the two sides of the substrate window. For example, photons emitted by the biofilm at  $45^{\circ}$  undergo a deflection of approximately  $13^{\circ}$  when penetrating the UV transparent substrate. Finally, the effective detection efficiency field for a given set-up was calculated by multiplication of the light cones of the excitation light source (given by the excitation field) and the acceptor cone of the optical fibers (given by the collection light field).

*Inclination angles* - Typical longitudinal cross-sections through this detection efficiency field are illustrated in Table 2.3 for different inclination angles of the optical fibers. With a



fiber configuration with low inclination angles ( $20^\circ/20^\circ$  for inner/outer fiber ring), the most efficient fluorescence detection would be below the substrate. Of course, there are no emitting fluorophores below the substrate such that this configuration yields an overall low biofilm collection efficiency (BCE) value ( $<0.4$ , see Figure 2.3. and Table 2.3). In contrast, with large inclination angles ( $70^\circ/70^\circ$  for inner/outer fiber ring) the most efficient detection would occur well above the substrate ( $\text{BCE} \sim 0.4$ ) and, even more, at biofilm level the detection efficiency pattern at biofilm level (see corresponding meridional cut in Table 2.3) is far from being uniform. As already outlined in Paper 1, best performance is obtained for inclination angles of  $35^\circ/30^\circ$  for inner/outer fiber ring with  $\text{BCE} = 1$ .

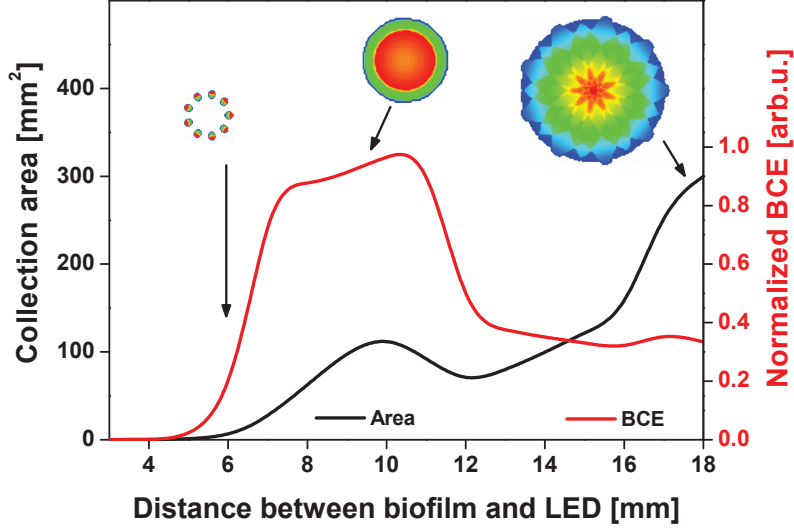
**Table 2.3.:** Simulation results for fluorescence detection efficiency pattern using different inclination angles for the detecting fibers. Red color corresponds to high and blue color to low detection efficiency.

Fiber angle (inner/outer)	Longitudinal cross-sectional	Meridional cross-sectional
$20^\circ; 20^\circ$		
$30^\circ; 25^\circ$		
$70^\circ; 70^\circ$		

*Sensor height* - The distance  $a$  between biofilm and LED is expected to have a considerable influence on the biofilm collection efficiency as well. As shown in Figure 2.7, the BCE and the



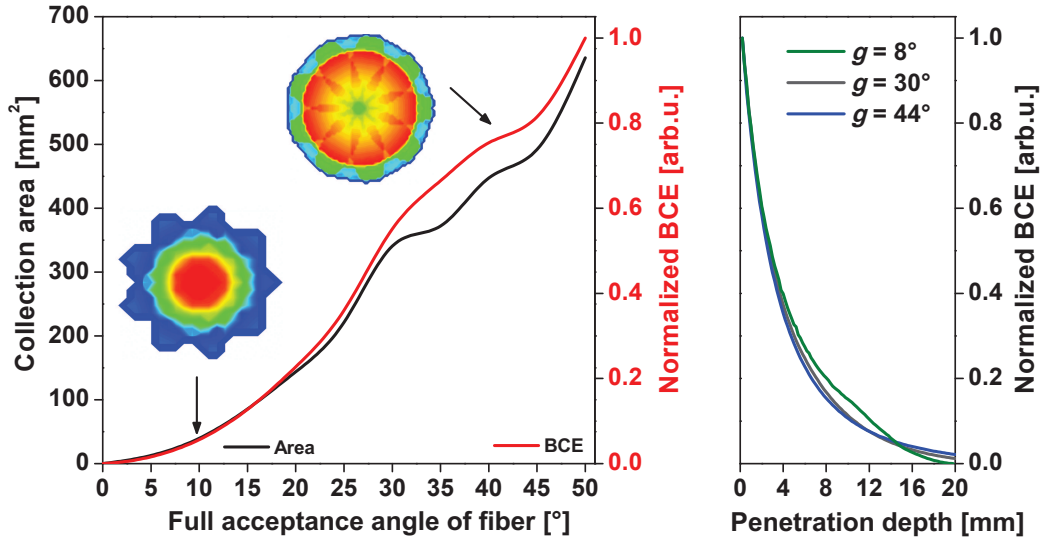
size of the collection area were calculated as a function of the distance between the biofilm and the excitation light source. As expected, the BCE decreases with distance to the excitation light source. As seen in the insets of the graph, the calculated fluorescence collection area increased at biofilm level on the substrate, however an optimum was reached at  $a = 10.6$  mm in terms of size of the detection area and efficient fluorescence collection.



**Figure 2.7.:** Collection area and normalized BCE as function of distance between biofilm and excitation light source. The data points were spline interpolated and BCE values have been referenced to the optimum value set to BCE = 1. The values in Table 2.2 were used as model parameters. Insets show the corresponding meridional cut through the fluorescence detection efficiency at biofilm level of which red color corresponds to high and blue color to low detection efficiency.

Owing to technical practicability by means of water pressure resistance of the optical window ( $b = 3$  mm), the dimension of the UV-LED (distance between LED protection window and light emitting semiconductor layer is 4 mm), the thickness of the excitation filter (2.6 mm) and a dense packing of the optical components, the minimum possible realistic distance is  $a = 11$  mm, hence close to the optimum value.

*NA of optical fibers* - The numerical aperture (NA) defines the product of refractive index and sine of the half acceptance angle of the fiber core. For this model all parameters were kept constant as used in the previous simulation run, however, the full acceptance angle of the fluorescence collecting fibers was increased stepwise. In Figure 2.8 the biofilm collection efficiency and the detection area are plotted against full acceptance angle of fiber and height dependence of the collected fluorescence.

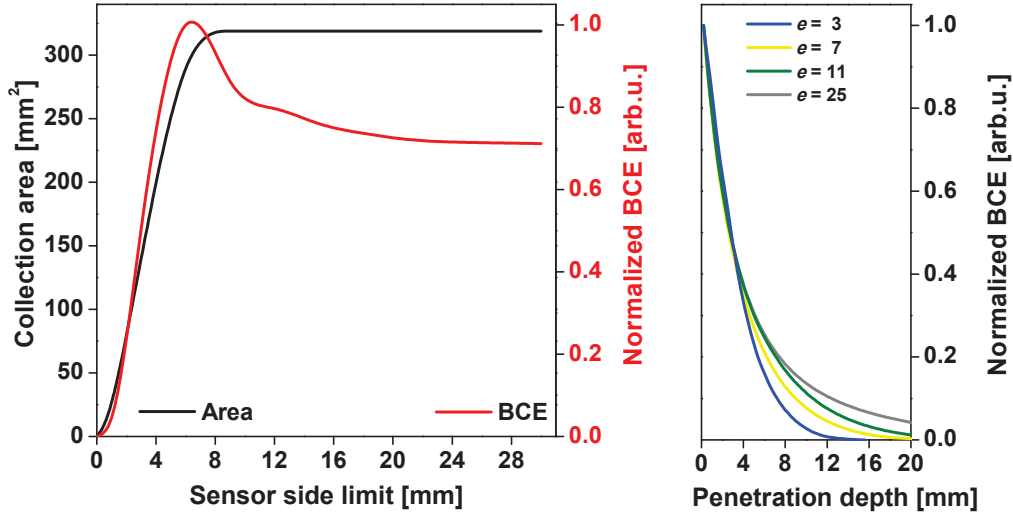


**Figure 2.8.:** Left: Plot of collection area (black curve) and normalized BCE (red curve) as function of different full acceptance angles of optical fibers. The insets illustrate the corresponding meridional cut through the fluorescence detection efficiency field at biofilm level and the red color corresponds to high and blue color to low detection efficiency. Right: The green, gray and blue curves represent the height dependence of fluorescence light collection for different acceptance angles of the fibers. The data points were spline interpolated and BCE values have been normalized. The values in Table 2.2 were used as model parameters.

The biofilm collection efficiency and area increase virtually quadratically with enlarging acceptance angle of the optical detection fibers. It can be seen in the inset images of Figure 2.8 for low acceptance angles that the area of fluorescence collection is only about  $40 \text{ mm}^2$  and exhibits a rather uniform spatial distribution of the intensity. With increasing acceptance angles of the fibers, the detection area increases as well. However, the fluorescence detection at the biofilm level is inhomogeneous. Moreover it has been checked if the height dependence of the collected fluorescence depends on the selected fiber NA value (right plot in Figure 2.8). Obviously, the penetration profiles slightly depend on the NA. Therefore, it can be concluded that wide acceptance angles of the fibers are important for sensitive fluorescence collection. The NA of the used fused silica fibers had a value of 0.26 resulting in a full acceptance angle of  $30^\circ$ . This is already at the higher end of technologically feasible multimode fibers ( $0.05 < \text{NA} < 0.4$ ), however, somewhat higher NA of up to 0.7 may become feasible by so-called photonic crystal fibers in the near future.

*Side limit* - The influence of the side limit  $e$  has been evaluated by simulating different diameters of the substrate area while keeping the other parameters constant (see Table 2.2). As shown in Figure 2.9, the optimum radius for a side limit is approximately 7 mm to achieve the maximum of BCE. The detection area increases more or less linearly to a maximum value

of  $320 \text{ mm}^2$  and of course, the excitation value depends on the chosen geometry of the sensor head including the NA of the fibers. At too high side limits, the BCE values start to decline. This can be explained by the height dependence of the collected fluorescence as seen in the right plot of Figure 2.9. If the side limit is too far, the fluorescence light is collected not only from the substrate area, but also from higher liquid levels above the substrate causing a decrease in the BCE.

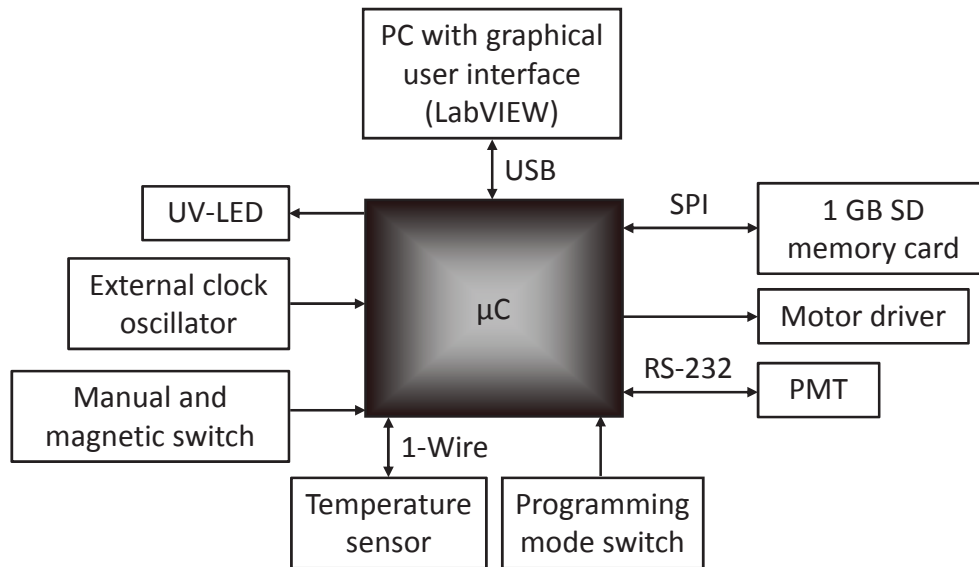


**Figure 2.9.:** Left: Plot of collection area (black curve) and normalized BCE (red curve) as function of different sensor side limits. Right: The blue, yellow, green and gray curves represent the height dependence of fluorescence light collection for different sensor side limits. The data points were spline interpolated and BCE values have been normalized. The values in Table 2.2 were used as model parameters.

In summary, the most significant influence on the biofilm collection efficiency can be attributed to the inclination angles of the optical fibers and the NA of the detection fibers. Most parameters of the biofilm sensor prototype could be chosen in a way that the computed BCE value is close to its optimum value.

## 2.5. Electronic Setup for the Biofilm Field Sensor

To run the biofilm sensor as data logger in the field, a home-made control electronic has been developed and assembled. A block diagram of the biofilm sensor system is shown in Figure 2.10.



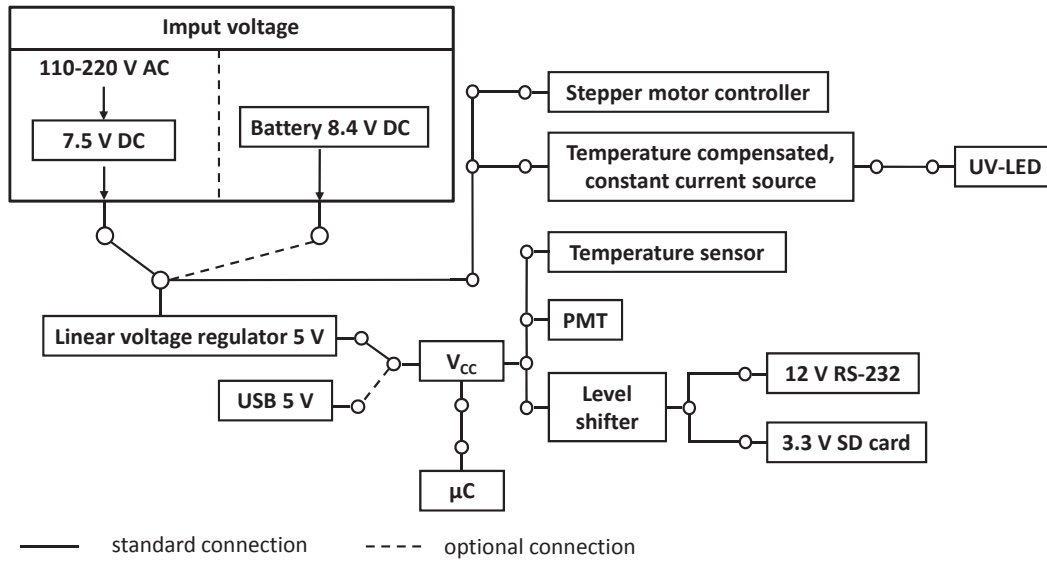
**Figure 2.10.:** Hardware block diagram of biofilm sensor system. Arrows denote the direction of data transfer.

Each block performs a particular function and illustrates how they are connected to each other. Since the central processing unit (CPU) is one of the major components that controls all the operations of a sensor unit, it has to be chosen carefully. The core of the biofilm sensor is a low-power complementary metal oxide semiconductor (CMOS) 8-bit microcontroller ( $\mu C$ ) which is a single integrated circuit containing processor core, memory, and programmable input/output peripherals based on enhanced reduced instruction set computer (RISC) architecture (Atmel, USA, ATmega8515). The key features of this  $\mu C$  are the ultra-low power consumption (less than 0.06 W in active mode and about  $6.5 \times 10^{-5}$  W in power-down and stand-by mode), the 8 kByte on-chip flash memory, the external and internal interrupt sources, and a programmable watchdog timer with separate on-chip oscillator. Other helpful features of the ATmega8515 are the joint test action group (JTAG) interface which allows reconfiguration of the programmable logic and a dual universal asynchronous receivers/transmitters (UARTs) controller that translates data between parallel and serial forms which are essential for communicating with other system components.

An external 7.4 MHz quartz oscillator provides a stable clock signal as well as internal device

timing and is used to specify the operating frequency for the  $\mu\text{C}$ . For biofilm fluorescence measurements, the  $\mu\text{C}$  activates the motor driver for closing a shutter and powering on the UV-LED. Thereafter, the photomultiplier tube (PMT), which is connected to a serial port (RS-232), detects the fluorescence intensity and the resulting data signals are delivered by a serial peripheral interface (SPI) and are stored on a secure digital (SD) memory card. The fluorescence measurement can be launched manually by either pressing a mechanical switch when the waterproof housing is removed or contact-free with a magnet when the sensor is used in the field. The timing, settings, and frequency of biofilm detection can be controlled by a graphical user interface (GUI) via universal serial bus (USB) 2.0 from a personal computer (PC). The GUI is discussed in more detail in Section 2.6. The programming mode switch has to be activated while the sensor is connected to a PC to enable changes in the biofilm sensor logger settings. A high-precision 1-Wire digital thermometer (Maxim, USA, DS18S20) able to measure temperatures from  $-10^\circ\text{C}$  to  $+85^\circ\text{C}$  with an accuracy of  $\pm 0.1^\circ\text{C}$  takes a measurement after each detection cycle of biofilm fluorescence.

As power supply for the biofilm sensor unit, a 110 - 230 V universal AC adapter or seven 1.2 V, 4500 mA nickel-metal-hydride rechargeable batteries may apply. The block diagram of the power management is shown in Figure 2.11.

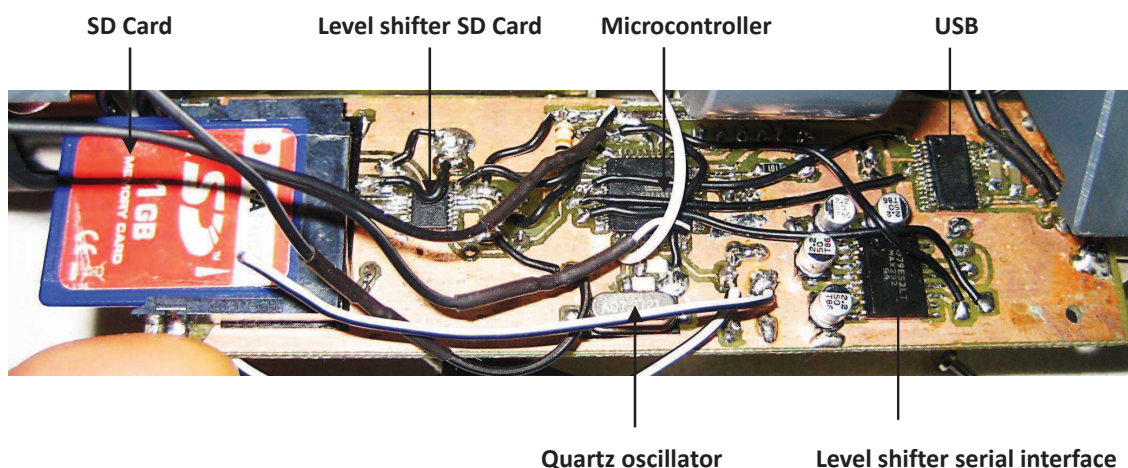


**Figure 2.11.:** Block diagram of the power management circuitry of the biofilm field sensor.

Highlighted are the temperature compensated high-precision constant current source with a precision of 0.02 % (Linear Technology, USA, LT3092) for UV excitation of the biofilm and the PMT, which is connected to a +5 V ( $V_{CC}$ ) linear voltage regulator (ST microelectronics,

USA, L78S05).

The electronic setup of the detector that includes a 1500 V high voltage step up DC/DC converter and a communication interface was already integrated in the PMT and developed by Hamamatsu, Japan. As level shifter of the communication ports of the  $\mu\text{C}$  a dual driver/receiver with  $\pm 12$  to  $\pm 5$  V (Maxim, USA, Max232) and a 3.3 V level shifter (Philips Semiconductors, NL, 74HCT365) have been used for SD card communication. A photograph of the electrical circuitry is given in Figure 2.12.

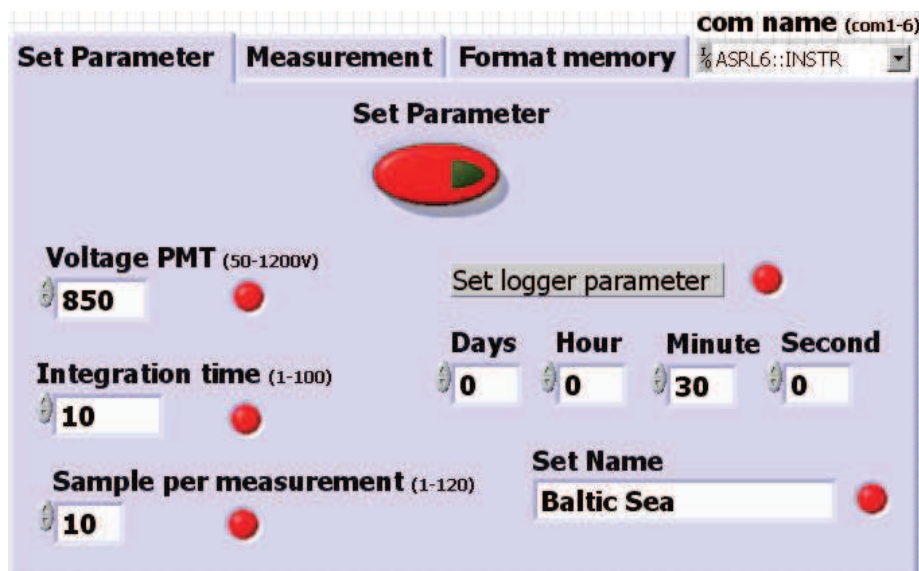


**Figure 2.12.:** Photograph of assembled main conductor board of the biofilm sensor.

## 2.6. Graphical User Interface

The GUI allows users to interact with the biofilm sensor via USB 2.0 to a standard PC and is developed using a home-made LabVIEW (National Instruments, USA) program. The GUI front panel allows the operator to control and program the measurement parameters such as voltage and integration time of the PMT of the biofilm sensor and is displayed in Figure 2.13. The interface contains three primary display areas for the user to select. In interface mode, the user controls the standard settings of biofilm fluorescence detection such as the voltage of the PMT and the integration time. Longer integration times correspond to higher sensitivities of the detector. These values have to be adjusted depending on the background fluorescence of the aquatic environment. Moreover, the number and frequency of the measurements may be set, ranging from a few seconds to several days depending on the experimental duration. The set up parameters are stored on the  $\mu\text{C}$ , however, the electronics is designed such that it is capable of operating in a portable system without any PC connection. The second user panel is designed to start a measurement from the PC which is useful for calibration measurements in the laboratory and the third one to delete all data from the memory card. The biofilm

fluorescence data read out from the memory card provides all necessary information such as fluorescence intensity, background fluorescence intensity and dark counts, detection date and time, and settings of the PMT.



**Figure 2.13.:** Screen capture of the graphical user interface display of the main window of the biofilm sensor.

# Paper II

## 2.7. Field Sensor for *in situ* Detection of Marine Bacterial Biofilms

*Novel sensor concept enables time-resolved detection of bacteria from initial attachment to mature cell clusters*

M. Fischer<sup>a</sup>, M. Wahl<sup>a</sup> and G. Friedrichs<sup>b,\*</sup>

<sup>a</sup> GEOMAR | Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

<sup>b</sup> Institute of Physical Chemistry, Christian-Albrechts-University Kiel, Kiel, Germany

\* To whom correspondence should be addressed.

E-mail address: [friedrichs@phc.uni-kiel.de](mailto:friedrichs@phc.uni-kiel.de) (G. Friedrichs).

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Contribution of M. Fischer

- Development and assembly of biofilm sensor
- Experimental work and data evaluation
- Preparation of the raw version of the paper

Contribution of Co-authors

- Supervision of the experimental work, joint design of sensor concept
- Completion of final paper



### 2.7.1. Introduction

IN marine environments nearly all surfaces become rapidly covered by microorganisms forming a biofilm. Following a biochemical conditioning phase, the biofilm formation starts within the very first minutes when pioneer bacteria cells adhere to the submerged surface {Wahl, 1989}. Marine biofilms cause serious technical problems by settling on ship hulls and their water conduits, navigational equipment, stationary port structures, industrial pipelines and tidal power plants. They cause severe damage by increasing the drag, roughness and friction resistance of submerged objects and accelerate biocorrosion of metals {Tamachkiarow and Flemming, 2003}. In natural ecosystems bacterial biofilms play more differentiated roles by enabling or preventing further biofouling by micro- and macroorganisms and serve as a unique living habitat {Harder, 2009}. Biofilms on the surfaces of marine organisms may substantially change their ecology and well-being {Wahl et al., 2012}. In order to gain insight into biofilm formation kinetics and dynamics, continuous and *in situ* monitoring of biofilm establishment in the marine habitat is desirable but the required temporal and spatial resolution is difficult to achieve. This article briefly outlines the design, development, and field application of a novel bacterial biofilm sensor {Fischer et al., 2012}.

### 2.7.2. Biofilm sensor concept

For marine applications, sensor requirements differ considerably from those for highly sophisticated laboratory instrumentation. The aim was to develop a robust and reliable ready-to-use sensor that allows the detection of biofilm formation dynamics *in situ*, online and non-destructively in the marine environment. The sensor concept should allow for autonomous operation with an operational time over several months as well as selective detection of the biofilm, i.e. distinguishing between organic and inorganic material on the surface. Additionally, a sufficient penetration depth is desired to account for the three-dimensional structure of biofilm that typically constitute highly patchy cell clusters of up to several hundred micrometers in diameter {Dalton et al., 1996}. To ensure a representative sensor signal of the inhomogeneous biofilm, a large detection area on the order of a square centimeter is required while keeping a low detection limit and a wide dynamic range to quantify the entire growth range from initially adsorbed bacteria cells up to a complex biofilm community. The fact that all organisms contain natural intracellular fluorophores can be utilized for fluorescence-based detection methods as they provide high sensitivity and selectivity, fast response time, and the capability of monitoring large areas *in situ* without sample contact. The natural protein fluorescence of bacteria, stemming for instance from amino and nucleic acids, has long been known as indicator for biomass and metabolic activity {Arrage et al., 1995, Determann, 1998, Sohn et al., 2009}. At wavelengths in the ultraviolet (UV) range, intrinsic protein fluorescence

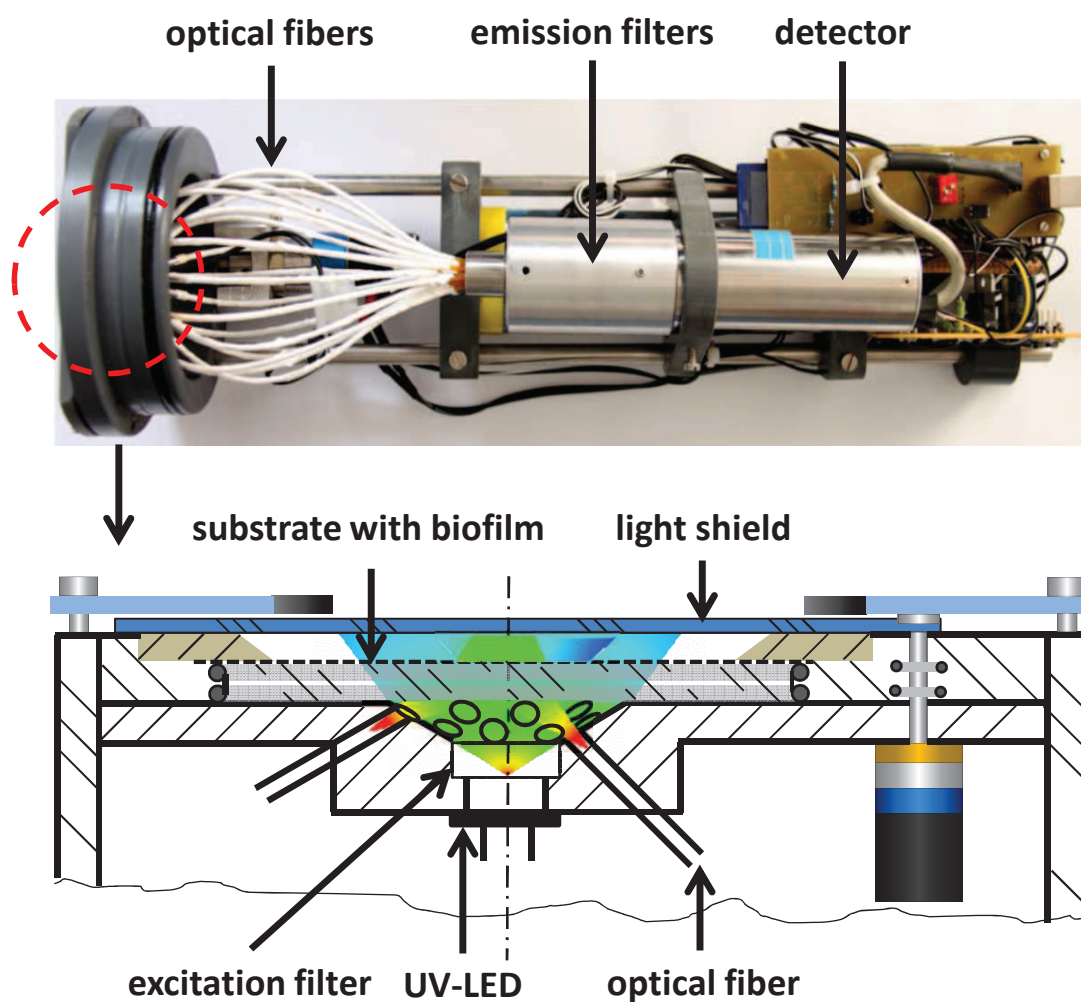
originates mainly from the three aromatic amino acids tyrosine, phenylalanine and tryptophan. Due to a very low quantum yield of phenylalanine and common quenching mechanisms of the emission of tyrosine, the native fluorescence in proteins is dominated by tryptophan. The indole chromophore of tryptophan can be selectively measured by optical excitation at a wavelength of 280 nanometers and detection of the corresponding peak fluorescence centered around 350 nanometers {Vivian and Callis, 2001}.

### **2.7.3. Field sensor layout**

The portable fiber optic probe for field application is contained in a waterproof housing (12 centimeters in diameter and 40 centimeters in length) that is deployable down to 50 meters water depth (Figure 2.14). The sensor head consists of a substrate for biofilm establishment, the light source with an excitation filter on top, the collecting fiber optics and a motor-driven cover plate. A UV transparent quartz window equipped with a gas permeable and low fluorescent foil for optimized bacteria growth is employed as settling substrate. The biofilm is back-illuminated and excited through the substrate using a 280 nanometer UV-light emitting diode (LED) in combination with a narrow bandwidth interference filter. Eighteen bundles of 30 optical fibers each are arranged hemispherically and in two rings around the LED for collection of the emitted fluorescence light. The tilt angle of the fibers is optimized for constraining the detected fluorescence volume to a layer close to the surface and for spatially uniform sensing of an effective area of approximately 0.5 square centimeters. At the end of the combined fiber bundles, the collected fluorescence emission is spectrally separated by a combination of two interference filters centered at 350 nanometers prior detection on a photomultiplier tube (PMT) operating in single-photon counting mode. The timing of the electronics, the readout of the detector, and the data recording on a 1 Gigabyte secure digital (SD) memory card is accomplished by a programmable microcontroller making the sensor package ready for use as a field data logger. A National Instruments Corp. LabVIEW-based graphical user interface (GUI) allows the user to control the sensor settings including the timing and sampling interval via universal serial bus (USB). Either a universal AC adaptor or seven nickel-metal-hydride rechargeable batteries with charge capacities of 4,500 milliampere hours at 1.2 volts could power the biofilm sensor unit.

### **2.7.4. Biofilm measurement sequence**

In order to measure the intrinsic fluorescence intensity of a marine biofilm under field conditions, a light shield in the form of a black anodized cover plate is closed to prevent the incidence of direct sunlight. The remaining background intensity is measured five times and its averaged value is subtracted from the total fluorescence intensity. To ensure a stable light output, the UV-LED is illuminated one second before launching the subsequent biofilm



**Figure 2.14..** Top: A photograph of the developed biofilm sensor without waterproof housing. Bottom: Schematic longitudinal cross-sectional view of the cylindrical sensor head with the main components and a color-coded region illustrating the excitation of the UV-LED and fluorescence detection efficiency pattern.

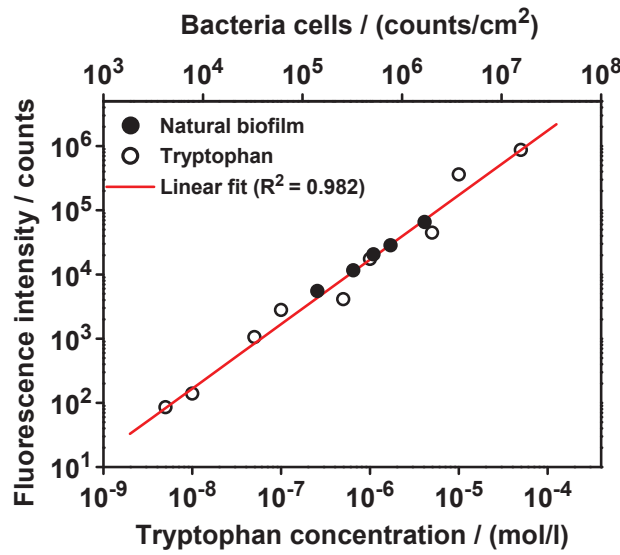
detection sequence. Typically, five fluorescence measurements with integration times of 10 milliseconds each are performed. Supplementary, a high-precision digital thermometer takes a measurement after each detection cycle. The background and total fluorescence intensity values, the setup parameters of the biofilm sensor, the actual time, and the temperature are recorded on the SD memory card. Possible damage to the DNA of the microbial community, which is expected to occur for a prolonged UV exposure {Elasri and Miller, 1999}, is prevented by switching off the UV-LED immediately after the fluorescence measurements. Finally, the light shield opens to allow for biofilm formation under natural light conditions. A complete measurement cycle takes about 12 seconds. Repeated measurements at preset intervals, typically every 15 - 60 minutes over time periods of several weeks, yield quasi-continuous sampling of the biofilm growth dynamics.

### **2.7.5. Detection range**

The sensor performance has been tested in the laboratory in April 2010 by placing a UV transparent cell culture dish on the sensor head filled with L-tryptophan solution in artificial seawater. Dilution series in the concentration range of  $5 \times 10^{-9} \leq c \leq 1 \times 10^{-4}$  moles per liter yield linear correlations between sensor signal and tryptophan concentration demonstrating the wide dynamic range from the nanomolar to the millimolar range (Figure 2.15). Further calibration measurements in combination with epifluorescence microscopic counts of bacteria cell numbers with two common marine bacteria strains, namely *Pseudoalteromonas carrageenovora* and *Bacillus subtilis*, and a natural bacterial biofilm community confirmed a low detection limit of about 4000 bacteria cells per square centimeter {Fischer et al., 2012}. Taking into account geometrical factors of the sensor, it can be concluded from these measurements that approximately thirty million tryptophan molecules per cell contribute to the measured signal, which is in good agreement with reported protein content of bacteria {Neidhardt and Umbarger, 1996}.

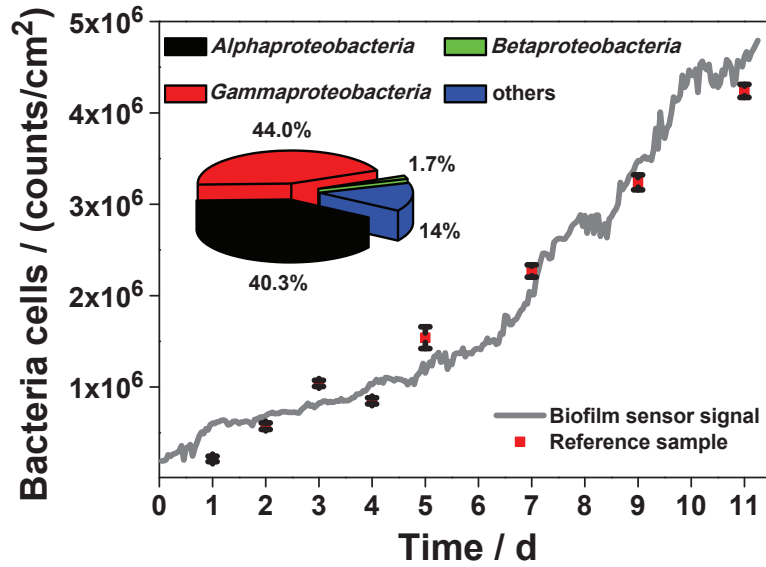
### **2.7.6. Field experiment**

The field sensor prototype was applied hourly to quasi-continuously monitor biofilm formation dynamics on a ship cruise in December 2011 about two nautical miles south of Hawaii. The sensor unit was installed on deck of the vessel in an outdoor mesocosm with steady exchange of Pacific Ocean water. Over 11 days, the intrinsic biofilm fluorescence intensity was measured (Figure 2.16) together with daily measurements of several physico-chemical water parameters. The average water temperature was 25.2°C, the salinity was 53.5 millisiemens per centimeter, the pH value was 8.2, and the oxygen content was 5.8 milligrams per liter. Reference settling substrates were placed inside the mesocosm under same hydrodynamic conditions to quantify the corresponding accumulated bacterial cell density of the biofilm.



**Figure 2.15.:** Double-logarithmic plots indicating the linear dependence of the fluorescence intensity with tryptophan concentration and bacteria cell number.

The bacteria were stained by a DNA-binding fluorescent dye, DAPI, and were quantified daily by epifluorescence microscopy. Twenty random images of the substrate were captured, and the bacteria cell numbers were counted by an ImageJ software program {Schneider et al., 2012}. The sensor readouts revealed an exponential trend in marine biofilm growth. Nevertheless, with 2.5 percent, even after 11 days, the area covered by the biofilm remained quite small. Moreover, it has been observed from the microcopy images that the biofilm community of the Pacific Ocean was dominated by bacteria, as the average cell size was only 0.83 micrometers. For a more detailed assessment of the December 2011 experiment, the relative abundance of major bacterial groups was accomplished by fluorescence *in-situ* hybridization (FISH) {Amann et al., 1990}. Common bacterial oligonucleotide probes revealed that the bacterial biofilm composition was dominated by *Gammaproteobacteria*, which a GAM42a probe {Manz et al., 1996} detected to be about 44 percent of biofilm; *Alphaproteobacteria*, which an ALF968 probe {Glöckner et al., 1999} detected to be about 40 percent; and *Betaproteobacteria*, which a BET42a probe {Manz et al., 1996} detected to be less than 2 percent (inset in Figure 2.16). Overall, a strong linearity,  $R^2 = 0.93$ , has been found between bacterial cell numbers of the reference subsamples and the biofilm sensor fluorescence data. This also demonstrates that natural bacterial films in the field, despite their changing and heterogeneous composition, exhibit linear signal response and that tryptophan fluorescence can be used as a universal measure for bacterial abundance. Between 2010 and 2011, other test experiments have been performed in the Baltic Sea with longer settling periods. These



**Figure 2.16..** Monitoring of bacterial biofilm growth in Pacific Ocean water (solid line). Analysis of bacteria cell density using DAPI-stained subsamples by optical microscopy (squares). Error bars represent standard error means ( $n = 20$ ) of the bacterial cell density. The inset shows average percentages of dominant bacterial groups.

experiments yielded a saturation level of bacterial coverage and partly revealed diurnal signal patterns. These results show that interesting new aspects of biofilm formation dynamics wait to be uncovered, and their detailed investigation requires the sensor's quasi-continuous measurement capabilities.

### 2.7.7. Conclusion

The developed sensor enables the detection of bacterial biofilms in the marine environment by detecting intrinsic tryptophan fluorescence of bacteria. It has been shown that biofilm fluorescence excited by a UV-LED at a wavelength of 280 nanometers and detected at 350 nanometers reveals a linear relationship between fluorescence intensity and bacteria cell number. The sensor was calibrated with two marine bacteria strains. The developed sensor enables the detection of bacterial biofilms in the marine environment based on detecting intrinsic tryptophan fluorescence of bacteria. It has been shown that biofilm fluorescence excited by a UV-LED at a wavelength of 280 nanometers and detected at 350 nanometers reveals a linear relationship between fluorescence intensity and bacteria cell number. The sensor was calibrated with two marine bacteria strains. A detection range of approximately four thousand to fifty million cells per square centimeter enables monitoring of biofilms from

initial attachment of bacteria cells up to fully developed complex biofilms. The results of the field experiment represent the first quasi-continuous dataset of bacterial biofilm establishment in the Pacific Ocean. Moreover, the flexibility, robustness and sensitivity of the sensor offer a high potential for applications in marine industry, biotechnology, and life sciences. Further upgrades of the sensor will allow simultaneous recording of biomass and metabolic activity of bacteria opening up novel applications.

# Marine Biofilm Formation

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### 3.1. Marine Surface Colonization

IN marine habitats, almost all living and non-living submersed surfaces become rapidly colonized by bacteria, fungi, diatoms, protozoa, invertebrate larvae and algal spores {Dürr and Thomason, 2010, Wahl, 1989, Zobell and Allen, 1935}. The most subtidal and intertidal solid surfaces are covered by biofilms including tissues of organisms from animals and plants, sediments, rocks and wood. Most microorganisms can form biofilms and 99% of the bacteria live in such a cell cluster {Costerton et al., 1987, Flemming, 2002}. Bacterial colonization of sediments and particles in the ocean are important components in the turnover, decomposition, and sinking flux of organic and inorganic matter and biological activity of these microbes play pivotal roles in transforming and degrading these aggregates. Moreover, 70% of the marine bacteria are mobile and exhibit chemotactic behavior which enables them to colonize organic matter within a few seconds, and attached bacteria grow 50% faster when clustering within nutrient patches around detrital particles {Grossart et al., 2003}.

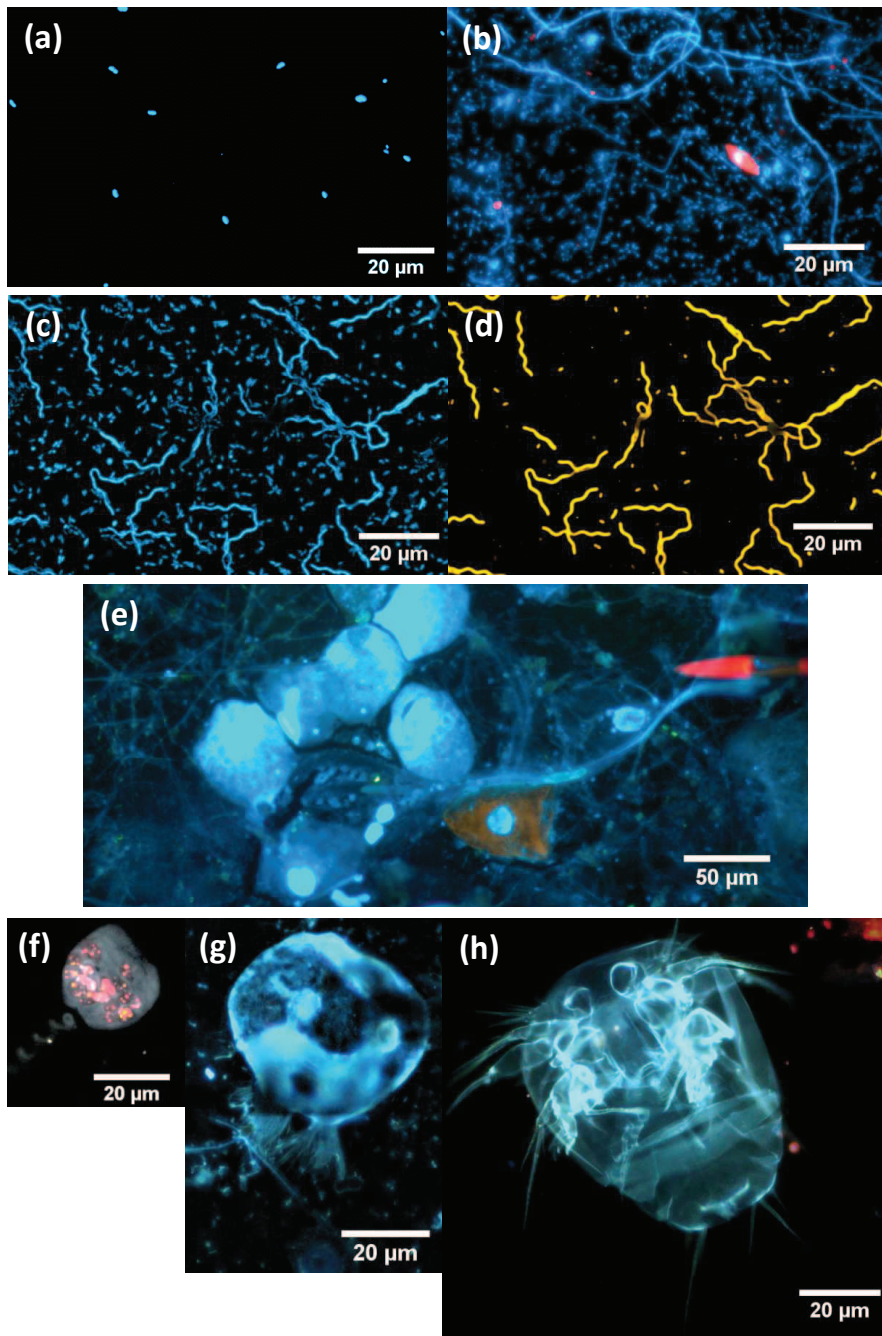
Not only organic particles are covered by microorganisms but also living surfaces such as cyanobacteria {Grossart, 2010}, macroalgae {Lachnit et al., 2010}, zooplankton {Grossart et al., 2010}, soft corals {Ainsworth et al., 2006} and ascidians {Wahl, 1995}. Such interaction between a basibiont (host) and an epibiont (on-growing organism) is known as epibiosis {Wahl, 1989} and has been recently reviewed by Wahl et al. {2012} and Steinberg et al. {2011}. As bacterial biofilms formed 3.43 billion-year ago in the aquatic environment {Allwood et al., 2007} this host specific microbe interaction must be a synergistic and symbiotic life. From the host perspective, exchange processes such as nutrients, gases, excretion of metabolites, or wettability of the surface will be affected by the epibacterial biofilm {Wahl, 2008}. However, bacterial biofilms are also known to have a beneficial effect on the host by accumulating nutrients from the environment {Decho, 2000}, providing it with vitamins {Croft et al., 2005, Matsuo et al., 2005}, absorbing UV radiation, and protecting the host with a first basic immune system. Like a "second skin" {Wahl et al., 2012}, biofilms can control subsequent colonization of the host {Nasrolahi et al., 2012, Wieczorek and Todd, 1998} and symbiotic microorganisms may defend their host against pathogen infections after a common evolution over millions of years. Up to date, very little is known about this host-microbe interaction and the effects at the ecological levels of populations, communities, and ecosystems which wait to be discovered in a new field of research named neuroecology {Derby and Aggio, 2011, Nevitt, 2011, Steinberg et al., 2011}. Also the biofilm benefits from epibiosis as favorable niches and settling substrates are limited in the aquatic environments. By settling on macroalgae, the host provides a substratum that is rich in organic material, minerals and secondary metabolites {Delille et al., 1997}. Hitchhiking on migrating organisms such as fish, jellyfish or zoo- and phytoplankton allow bacteria to travel and cross aquatic boundaries

(thermo-, chemo-, and pycnoclines) that are otherwise impassable for the bacteria {Grossart, 2010}.

Marine substrate colonization consists of the following processes {Characklis, 1981, Railkin et al., 2004, Wahl, 1989}:

- Immediately after contact with seawater, a biochemical conditioning of the surface occurs by adsorption of macromolecules and ions.
- Within the first hour the reversible and irreversible bacterial attachment colonization commences.
- During the first week the surface is colonized by unicellular eukaryotes such as diatoms, yeast and protozoa.
- Invertebrate larvae such as barnacles, mussels, polychaetes, bryozoans, tunicates and coelenterates, and spores of macroalgae follow within a lag of one to several weeks.

Biochemical conditioning of a submersed surface starts immediately with the adsorption of macromolecules, ions and other dissolved organic substances. The layer consists of sugars, amino acids, proteins, fatty and humic acids and facilitates the settlement of motile bacteria {Fletcher and Loeb, 1979, Pringle and Fletcher, 1986}. Zobell {1943} reported that in the marine habitat adherence of bacteria in phase I is reversible as well as irreversible. Further investigations showed that biofilm formation is initiated by attachment of specific groups of planktonic bacteria in seawater. The composition of bacterial communities varying over the first hours to days has been characterized as the sequence of a pioneer-driven accumulation of biomass followed by an enrichment of other groups {Jones et al., 2007, Lee et al., 2008}. The following phase II is characterized by a net accumulation of microorganisms due to exponential cellular reproduction followed by a detachment, dispersal and sloughing phase III. The bacteria in most biofilms produce extracellular polymer substances (EPS) that provide a protective and stable microenvironment against fluctuating conditions. The EPS are biopolymers that comprise mainly polysaccharides, proteins, lipids and nucleic acids. Biofilm organisms can maintain stable arrangements of synergistic microconsortia of different species and, thus orchestrate the degradation of complex substrates {Flemming, 2002}. Nutrients from the environment can accumulate in the matrix and ensure survival under oligotrophic conditions and provide protection from a variety of environmental stresses such as UV radiation, pH shifts, osmotic shock, and desiccation {Decho, 2000, Lee et al., 2003}. Marine biofouling is usually regarded as a sequence of accumulation and growth of several organisms, however, it involves the same processes: transport to the surface, settlement, attachment, growth and detachment for each settling community {Railkin et al., 2004}. Figure 3.1 demonstrates a typical fouling sequence observed in the Baltic Sea by applying epifluorescence microscopy.



**Figure 3.1.:** Epifluorescence micrographs of microorganisms in Baltic Sea biofilms. Images (a-c, e-h) were stained with a fluorescence dye DAPI. Images show: (a) three days old bacterial biofilm, (b) biofilm with red fluorescent diatoms, (c) twelve days old bacterial biofilm and (d) the same microscope field of view as in (c) hybridized with CY3-labeled probe CF319a for *Cytophaga*-like bacteria staining. Image (e) shows a 30 days old complex biofilm which is composed of a complex multispecies microbial community, i.e., bacteria, diatoms, ciliates (f,g), and (h) nauplius larvae of *Balanus improvises*.

### 3.2. Mathematical Description of the Marine Biofilm Fouling Sequence

Generally, a Gompertz function  $g(t)$  is a mathematical model for a time series in which growth is slowest at the beginning and end of a time period. It can be described as follows:

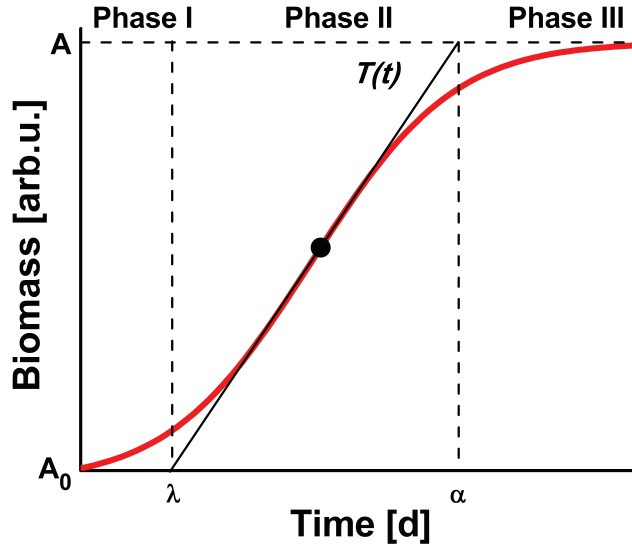
$$g(t) = Ae^{be^{ct}} \quad (3.1)$$

and  $b, c$  are negative numbers. The upper asymptote  $A$  is set by  $Ae^{be^{-\infty}} = Ae^0 = A$ ,  $b$  sets the  $y$  displacement,  $c$  the growth rate and  $e$  is the Euler number. To estimate the inflection point of the sigmoidal function, at  $t = t_i$ , the second derivative is set to zero:

$$\frac{d^2g}{dt^2} = 0 \rightarrow t_i = \frac{b}{c} \quad (3.2)$$

For reparameterization of the growth model, as illustrated in Figure 3.2, the tangent  $T(t)$  through the inflection point is described by:

$$T(t) = \mu(t - \lambda) + A_0 \quad (3.3)$$



**Figure 3.2.:** Sigmoidal growth curve of a bacterial biofilm with biological relevant parameters which are the maximum specific growth rate  $\mu$ , the induction time  $\lambda$ , the end of the exponential time  $\alpha$ , the start value of the asymptote  $A_0$  and the end of the asymptotic phase  $A$  over a certain period of time (modified from {Zwietering et al., 1990}).

The point of time  $\alpha$ , which defines the end of the exponential net accumulation phase II and the beginning of the pseudo stationary phase III, can be written as:

$$\alpha = \lambda + \frac{A - A_0}{\mu} \quad (3.4)$$

Using these definition, the modified Gompertz equation can be rewritten with biologically significant parameters:

$$g(t) = (A - A_0) \exp \left\{ -\exp \left[ \frac{\mu e}{A - A_0} (\lambda - t) + 1 \right] \right\} + A_0 \quad (3.5)$$

The crucial biological parameters are the maximum specific growth rate  $\mu$ , the conditioning/induction time  $\lambda$ , the asymptotic level  $A$ , and the start value observed before the exponential growth phase  $A_0$ . The subsequent attachment, development and growth of unicellular and multicellular eukaryotes may also be described by a sum of several Gompertz functions according to:

$$G(t) = A_0 + \sum_{i=1}^n g_i(t) \quad (3.6)$$

with  $g_i(t) = (A_i - A_{i-1}) \exp \left\{ -\exp \left[ \frac{\mu_i e}{A_i - A_{i-1}} (\lambda_i - t) + 1 \right] \right\}$  over a certain period of time with integer  $i = 1, \dots, n$ .

# Paper III

## 3.3. On Biofilm Formation - Dynamics in Natural Marine Environments

M. Fischer<sup>a,\*</sup>, G. Friedrichs<sup>b</sup>, M. Wahl<sup>a</sup> and T. Lachnit<sup>c</sup>

<sup>a</sup> GEOMAR | Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

<sup>b</sup> Institute of Physical Chemistry, Christian-Albrechts-University Kiel, Kiel, Germany

<sup>c</sup> Centre for Marine Bio-Innovation, The University of New South Wales, Sydney, NSW, Australia

\* To whom correspondence should be addressed.

E-mail address: matthias.fischer@york.ac.uk

Manuscript in preparation for *Applied and Environmental Microbiology*.

Contribution of M. Fischer

- Development and assembly of biofilm sensor
- Experimental work and data evaluation
- Preparation of the raw version of the paper

Contribution of Co-authors

- Supervision of the experimental work
- Statistical analyses of the data
- Completion of final paper

### 3.3.1. Abstract

**A**NALYZING the dynamic of biofilm formation is indispensable to understand and control surface colonization in natural environments. While laboratory screening methods of biofilm formation are well established, studies in marine environment are so far based upon destructive methods and can only represent a snap shot of biofilm establishment. In order to explore biofilm formation dynamics under varying biotic and abiotic parameters in the marine environment, we applied a recently developed optical biofilm sensor to quasi-continuously analyze marine biofilm formation *in situ*. In combination with microscope assisted imaging we investigated biofilm formation from its beginning to mature multi-species biofilms. In contrast to laboratory studies on biofilm formation of selected bacterial strains a smooth transition from initial attachment to colony formation and exponential growth could not be observed in the marine environment. Initial attachment was rather followed by an adaptation phase of low growth and homogeneously distributed solitary bacterial cells. Within the first days of settlement we observed a diurnal variation of biofilm densities suggesting a transient state of biofilm formation driven by a circadian timing mechanism of bacteria. The presence of seaweed tremendously reduced the adaptation phase compared to control surfaces from four to only two days. This shortening of adaptation time in the presence of seaweed resulted in an earlier decrease of growth rate, leading to similar bacterial densities on treatment and control surfaces after 14 days.

# Impact of Marine Macroalgae on Bacterial Biofilms

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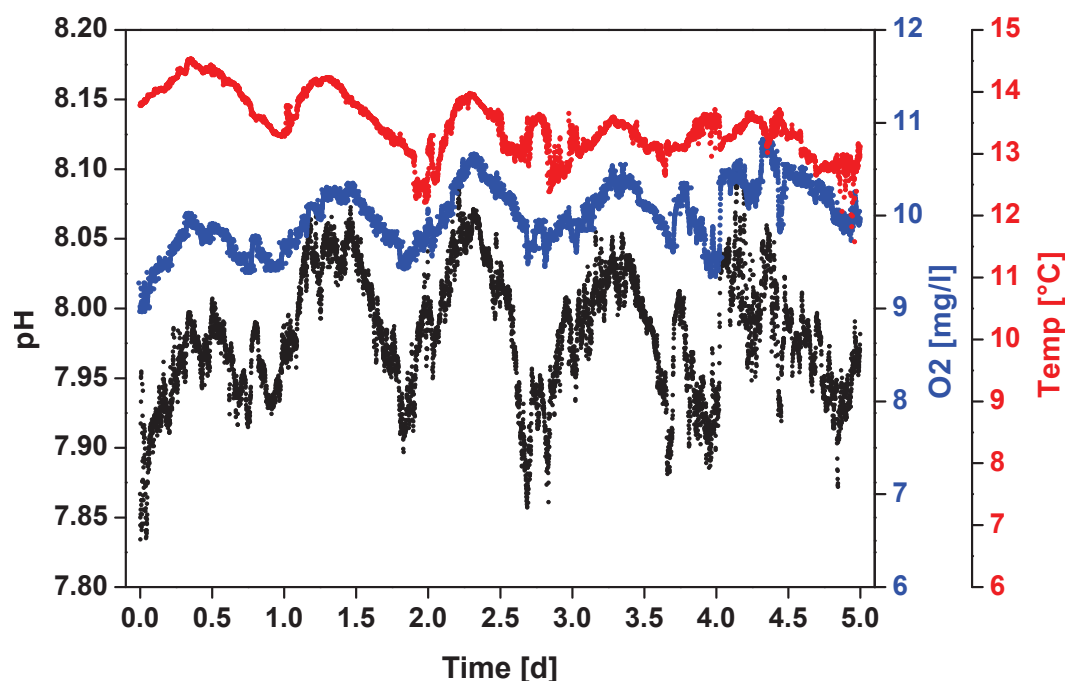


#### 4.1. Effects of macroalga on physical, biological and chemical water parameters

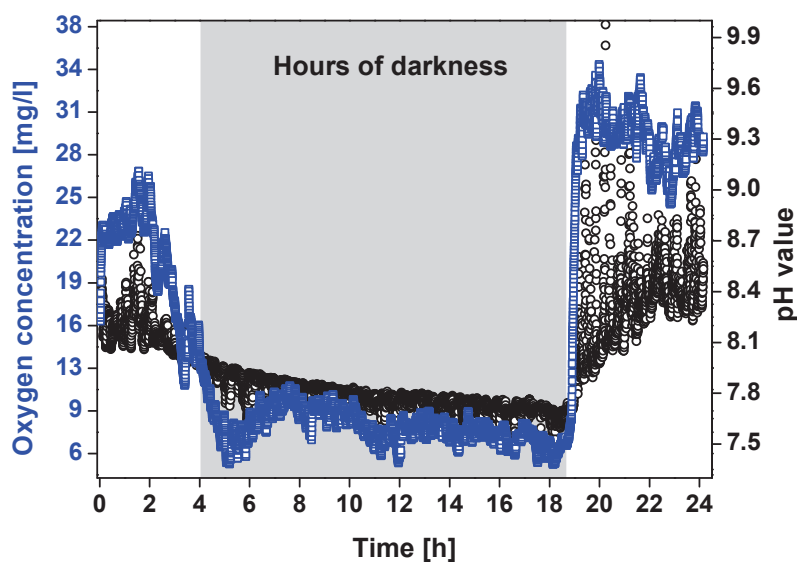
As discovered in Section 3.3 there is an influence of macroalgae on biofilm attachment and growth dynamics. Generally, the alga represents a safe micro-niche for microbial colonization and plays critical roles in structuring of the marine environment {Burke et al., 2011}. Due to their high metabolic activity, marine macroalga hold the potential to change the physical, biological and chemical parameters of their aquatic environment {Davis et al., 1983, Delille et al., 1997, Hollants et al., 2013, Mow-Robinson and Rheinheimer, 1985}. To explore possible factors affecting the interaction between biofilms and macroalgae, three main environmental parameters have to be considered and will be discussed as follows.

*Change of physical seawater parameter* – It is commonly known that during photosynthetic activity of seaweed the uptake of CO<sub>2</sub> causes a decrease in total inorganic carbon availability and an increase in pH. Moreover, during day time the algae play a key role in oxygen (O<sub>2</sub>) production and more than 200% saturation may occur {Krause-Jensen et al., 1999}. During the hours of darkness, algae consume O<sub>2</sub> for cell respiration whereby the oxygen concentration is significantly reduced. Previous studies have reported that in shallow-water *Fucus vesiculosus* habitats the diurnal oscillations in pH with pH 7.8 during night and pH 9.1 at day time are substantial {Middelboe and Hansen, 2007, Pearson et al., 1998, Saderne et al., 2013}. Of course the extent of observed pH changes depend on water current, and seasonal and diurnal variability in photosynthetic production. Moreover, Delille et al. {1997} observed a temperature gradient of 2 °C between the vegetated and unvegetated shallow coastal water. Figure 4.1 shows diurnal variations of pH, dissolved oxygen concentration and temperature. The measurements were carried out for five days in an outdoor mesocosm with permanent exchange of Baltic Sea water. The sensors were in close proximity to a field of the macroalgae *Fucus vesiculosus*.

In addition, to get a better understanding of the explored diurnal variations, a detailed examination of the diffusive boundary layer on the surface of *Fucus sp.* by Spilling et al. {2010} and Irwin and Davenport {2002} showed that the microhabitat is characterized by extreme variation of pH and dissolved oxygen concentration. The measurements were carried out by microsensors {Kühl and Revsbech, 2001} under artificial light conditions. To investigate the pH and O<sub>2</sub> under natural light conditions during a day cycle inside the boundary layer of *Fucus vesiculosus*, a similar experimental setup has been applied and the data were recorded every ten seconds. The results are plotted in Figure 4.2 and show a distinct diurnal variation in pH and O<sub>2</sub> concentration. The maximum pH measured was 9.8 and the maximum O<sub>2</sub> concentration was 33.8 mg/l during day time. In contrast, the minimum pH was 7.7 and the minimum O<sub>2</sub> concentration was 6.3 mg/l before sunrise.



**Figure 4.1.:** Quasi-continuous measurements of pH, dissolved oxygen concentration and temperature during five days in September 2010 starting at 8 a.m. The sensors were placed inside a field of *Fucus vesiculosus* plants in an outdoor mesocosm with continuous exchange of Baltic Sea water.



**Figure 4.2.:** Diurnal variation of oxygen concentration and pH value in the boundary layer of *Fucus vesiculosus*. The measurements were carried out under natural light conditions in October 2010 by the use of microelectrodes.

The bacterial biofilm community in the surrounding environment may be affected by the diurnal changes of physical water parameters as bacterial attachment and growth is known to be sensitive to small pH changes {Garrett et al., 2008, Krause et al., 2012} and O<sub>2</sub> {Turley and Lochte, 1986}.

*Change of biological and chemical seawater parameters* – Marine macroalgae provide environments rich in bioavailable nutrients, minerals and secondary metabolites {Delille et al., 1997, Goecke et al., 2010}. To date, it is not well understood how algal secondary metabolites affect different phylogenetic groups of a biofilm community. The release of organic carbon into the aquatic environment by macroalgae due to metabolic activity can be selective in its stimulation of bacterial growth {Bell, 1980, Brock and Clyne, 1984}. It has been reported that macroalga harboring a specific community differ qualitatively and quantitatively from their surrounding planktonic community {Bengtsson et al., 2010, Hempel et al., 2010, Lachnit et al., 2011}. Bacteria of the class of *Alphaproteobacteria* and from the genera *Rhodobacter* and *Roseobacter* were found as highly abundant group on *Fucus vesiculosus*. As discovered in Section 3.3, the biofilm community on artificial surfaces is dominated by *Alphaproteobacteria* during the first days when the brown alga *F. vesiculosus* is in close proximity. A possible explanation for this might be that bacteria from the surface of the algae establish a biofilm on the artificial substrate and can deal with the available nutrients release by the macroalgae. In conclusion, macroalga bioavailable nutrients can affect bacterial fouling communities by altering the community composition and by increasing the overall density of bacteria.

From the perspective of the algae, the chemical control of epibiotic bacterial colonization on their surface can be expected to be driven by pro- or antifouling compounds as reviewed by Goecke et al. {2010, 2012} and Wahl et al. {2012}. Only a few studies have investigated the interaction between biofilm formation and macroalgal chemical compounds in the marine environment {Lachnit et al., 2010, Persson et al., 2011}. Therefore, the interaction of surface associated chemical compounds of the macroalga *Fucus vesiculosus* and their response to a bacterial marine biofilm community in the field will be discussed in detail in the next Section 4.2.

# Paper IV

## 4.2. Compounds associated with algal surfaces mediate epiphytic colonization of the marine macroalga *Fucus vesiculosus*

T. Lachnit<sup>a,\*</sup>, M. Fischer<sup>b</sup>, S. Künzel<sup>c</sup>, J. F. Baines<sup>c,d</sup>, and T. Harder<sup>a</sup>

<sup>a</sup> Centre for Marine Bio-Innovation, University of New South Wales, Sydney, NSW, Australia

<sup>b</sup> GEOMAR | Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

<sup>c</sup> Max-Planck Institute for Evolutionary Biology, Plön, Germany

<sup>d</sup> Institute for Experimental Medicine, Christian-Albrechts-University of Kiel, Kiel, Germany

\* To whom correspondence should be addressed.

E-mail address: t.lachnit@unsw.edu.au (T. Lachnit).

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### Contribution of M. Fischer

- Joint settlement assays in the field and laboratory
- Quantitative analyses of the bacterial community
- ImageJ programming
- Completion of final paper

### Contribution of Co-authors

- Extraction and fractionation of the chemical compounds
- Qualitative analyses of the bacterial community
- Writing the paper

#### 4.2.1. Abstract

THE macroalga *Fucus vesiculosus* carries a specific community of surface bacteria. To identify chemical compounds that possibly mediate abundance and community composition of algae-associated bacteria, we tested the effect of surface-available algal compounds on bacterial settlement and community composition under field conditions. Compounds on algal thalli were separated from the surface by extraction with organic solvents and investigated on growth inhibition and settlement of bacterial isolates. Based on *in vitro* data, partially purified extract fractions were then exposed to bacterial colonizers *in vivo* followed by bacterial enumeration and community analysis. The polar fraction of the algal surface extract revealed a significant profouling effect for Vibrionales, whereas the nonpolar fraction - containing the xanthophyll pigment fucoxanthin and other unidentified nonpolar surface compounds - revealed a significant 80% reduction of surface colonizing bacteria. The analysis of bacterial surface communities by 454 pyrosequencing demonstrated that the antifouling activity of nonpolar algal surface compounds was targeting the abundance of natural bacterial colonizers rather than the relative composition of bacterial members within the community. Moreover, the bacterial community composition on *F. vesiculosus* was markedly different from artificial control substrates and chemically manipulated experimental treatments, suggesting that other, nonextractable surface features and/or physical properties render algal-specific epiphytic bacterial communities.

# Synthesis and Conclusion

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THE experimental results of this thesis contribute to a better understanding of patterns in marine biofilm formation dynamics. To the best of my knowledge, biofilm establishment has been investigated with an optical sensor *in situ* and in real time in the marine habitat for the first time. Section 2.2 starts with fluorescence measurements in the laboratory to verify the fluorescence wavelength of pure bacteria cultures. The results show that bacteria emit fluorescence light in the spectral range of 300–450 nm upon excitation in the UV range at 280 nm. The amino acid tryptophan dominates the intrinsic fluorescence signal of the bacteria, which is in agreement to the findings of Angell et al. {1993}. Based on this investigation, an optical fiber biofilm sensor has been developed. An important aspect of design was the arrangement of the entire optical system such as excitation light source, fluorescence collecting fibers, optical filters and detector. After comprehensive optical modeling, the inclination angle of the optical fibers turned out to be a key parameter for sensitive biofilm detection. As a result, a novel, biofilm specific and patent pending (M. Fischer, G. Friedrichs, M. Wahl, Großflächiger Biofilmsensor/Large-area biofilm sensor, DE 102011101934.4, see Appendix B) sensor head has been developed. Calibration measurements were carried out by using two marine bacteria stains and it has been shown that the sensor is capable to detect biofilms from the first attachment of bacteria cells to complex mature biofilms. The output signal of the biofilm sensor reveals a good linearity between fluorescence signal and cell number or surface coverage. The sensor is equipped with a data logging electronics as well as a waterproof housing and has been applied to the marine environment.

Considering the monitoring of initial net accumulation of biofilm on the sensor substrate with a temporal resolution of fifteen minutes, section 3.3 presents the first quasi-continuous *in situ* measurements of biofilm formation. Similar results in numbers of attached bacteria have been reported for other marine environments after 6 up to 48 hours {Becker, 1998, Dalton et al., 1996, Zobell and Allen, 1935} indicating that the initial settling dynamics follows a general pattern. Moreover, the section provides insights into biofilm establishment over time periods of several weeks and investigates the growth to mature microcolonies harboring multispecies. Biofilm formation in the Baltic Sea has been investigated under seasonally varying biotic and abiotic environmental conditions and revealed consistent seasonal differences. Depending on the microbial fouling community and settling pressure, which is seasonally variable in intensity and species composition {Andersson et al., 2010}, the accumulation and growth rate of biofilms show different behavior. The cell sizes of the adhering DAPI stained biofilm community have been analyzed by epifluorescence microscopy and show a strong seasonal variation between May and November. During autumn times, the average cell sizes are smaller and the coverage area is less than in spring. As benthic diatoms show a limited biological activity and growth pattern during autumn and winter {King and Schramm, 1976}, the biofilm community is composed mainly of bacteria. For a mathematical description

of the biofilm attachment and growth curves investigated by the biofilm sensor, a basic marine biofilm model has been developed. The model contains sigmoidal functions which are generally used to describe the growth of living organisms {Zwietering et al., 1990}. It could be shown that the model is suitable to describe *in situ* data of biofilm formation dynamics. Moreover, natural microbial biofilms are composed of a multitude of different organisms which settle in successive steps: initial reversible and irreversible net adsorption of bacteria, irreversible attachment and growth of bacteria and finally the attachment of diatoms and unicellular eukaryotic organisms (reviewed in {Railkin et al., 2004}). For each settling step, the specific growth rate and the beginning and end of these phases have been calculated by the model which is generally applicable for further considerations of biofilm dynamics. Owing to the high sensitivity of the developed biofilm sensor, it was possible to resolve fine scale diurnal variations of the biofilm fluorescence signal.

In section 3.3 it has also been explored that the macroalga *Fucus vesiculosus* has a beneficial effect on biofilm formation in the marine environment. Field investigations of the planktonic bacteria abundance in kelp-dominated ecosystems have indicated a 100-10,000 times higher bacterial abundance within this ecosystem {Davis et al., 1983}. At Kiel Fjord in 10 m depth, where the ecosystem is dominated by red algae, a higher abundance of free living bacteria has been observed {Mow-Robinson and Rheinheimer, 1985}. These facts indicate a higher fouling pressure which may result in a shift of bacterial settling dynamics and biofilm establishment during the experiment with *Fucus vesiculosus*. Moreover, the basic bacterial community composition changed when the brown alga was in close proximity. These results were obtained by staining biofilm samples after four and twelve days with rRNA targeting oligonucleotide fluorescent probes. The method only gives a general overview of the basic community structure and is depending on the metabolic activity and physiological state of the target organisms {Bouvier and Del Giorgio, 2003}. However, to obtain a first overview of major bacterial groups and following the same hybridization, image acquisition and analysis protocol for all samples, the data can be conveniently compared. The biofilm community structure on the reference samples is dominated by *Alphaproteobacteria* after four days when *Fucus vesiculosus* is adjacent.

Finally, in section 4.1 it has been observed that the macroalga *F. vesiculosus* carries a specific bacterial community on the surface and the most dominated classes are *Alphaproteobacteria* (see also {Lachnit et al., 2011}). This indicates that the bacteria associated with the macroalgae are able to settle on artificial substrates and are responsible for the initial steep rise of the biofilm sensor signal. Moreover, section 4.1 focuses on the ecological role of chemical compounds released by the macroalga, and their effect on the bacterial biofilm abundance and community structure in the marine environment. Macroalgae provide a settling substrate for bacteria rich in bioavailable nutrients which make them attractive for colonization {Delille



et al., 1997, Wahl et al., 2012}. On the other hand, the macroalgae have to control the surface colonization by the release of bioactive compounds as reviewed in Goecke et al. {2010}, Hollants et al. {2013}, Persson et al. {2011}.

Due to the beneficial effect on biofilm development, field experiments were carried out to identify chemical compounds that possibly mediate abundance and community composition of marine biofilms. Thereby, purified extract fractions of chemical compounds from the algal thalli were extracted {Lachnit et al., 2010} and tested *in vivo* followed by bacterial enumeration and community analysis by 454 pyrosequencing. The polar fraction of the algal surface extract revealed a significant profouling effect and significantly stimulated the growth of bacteria. Moreover, similar effects were investigated by Goecke et al. {2012} by testing extracts of sixteen macroalgae. It is commonly known that macroalgae release organic carbon into the aquatic environment due to metabolic activity and this nutrient can be selective in its stimulation of bacterial growth {Bell, 1980, Bengtsson et al., 2010, Brock and Clyne, 1984}. However, the non-polar fraction of the extracts containing the xanthophyll pigments fucoxanthin and other unidentified non-polar surface compounds revealed a significant reduction of biofilm establishment. This finding is in agreement with other studies investigating antibacterial macroalgae surface extracts {Saha et al., 2011, Viano and Bonhomme, 2009, Wahl et al., 2010}. It can therefore be assumed that the anti-settlement compounds play an important role inside the boundary layer of the macroalgae to control the colonization of their surface. However, the excreted compounds have a beneficial but selective stimulation effect of biofilm establishment in the surrounding marine environment. Taken together, these findings indicate that marine biofilm formation dynamics are a complex process in which several environmental factors are involved and more research is necessary to better understand the establishment in the marine habitat.

# Outlook

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**E**SPECIALLY in environments where it is difficult to collect samples regularly such as the abyssal zone of the ocean, it would be interesting to record biofilm formation quasi-continuously. The developed biofilm sensor holds potential for applications in aquatic sciences including rivers, lakes and deep sea research.

Moving towards higher flexibility in monitoring natural biofilms, the optical system has to be slightly modified. Spectroscopic investigations of various marine biofilms based on LED arrays operating at different excitation wavelengths will enable multichannel detection. Such an extension provides the opportunity for simultaneous measurement of biomass and microbial activity by means of the native fluorescence of tryptophan and NADP<sup>+</sup> {Tartakovsky, 1996, Wolf et al., 2001}. This may help to understand diurnal variations in the tryptophan fluorescence intensity. Further experimental investigations are needed to explore marine biofilm formation over time periods of three to six months. Therefore, the application of a reference channel for monitoring of the LED output intensity is recommended which will improve the accuracy of the biofilm sensor. An identification and classification of multiple species of the biofilm community will be feasible by measuring specific pigments such as xanthomonadins, erythromycin, pyocyanin and bacteriochlorophyll {Determann, 1998}. Moreover, the biofilm establishment in close proximity to macroalgae can be studied by field experiments with different marine macroalgae species in the marine environment. Furthermore, field investigations on the interaction of marine macroalgae and biofilm establishment may be carried out by a combination of continuous biofilm detection and controlled dispensing of seaweed surface extracts on a substrate.

For exploring the role of epibiotic biofilms on living substrates such as macroalgae, soft corals and ascidians; future sensor developments should focus on surface sensitive detection methods. For example, attenuated total reflection (ATR) sampling technique used in conjunction with Fourier transform infrared (FTIR)-ATR spectroscopy are used to study biofilms {Nivens et al., 1993, Schmitt and Flemming, 1998}. Typically, the penetration depth into the epibacterial film is only between 0.5 and 2  $\mu\text{m}$  and therefore a low background signal of the host can be assumed. Moreover, two-dimensional photonic crystals in photonic crystal fibers have been used to detect bacteria in very low concentrations {Li et al., 2013}. Photonic crystals are composed of periodic dielectric nanostructures that affect the propagation of electromagnetic waves and interferes with the sample on the surface {Krauss et al., 1996}. After further functionalization of the sensing element, photonic crystals sensors hold the potential for selective and label-free detection at the single cell level such as bacterial pathogens {Bonifacio et al., 2010}.

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# Eidesstattliche Erklärung

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Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation - abgesehen von der Beratung durch meine Betreuer - selbstständig verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel von mir eingesetzt wurden.

Des Weiteren versichere ich, dass die vorliegende Dissertation weder ganz noch zum Teil bei einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegen hat.

Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden.

Kiel, den 5. Dezember 2013

# Appendix

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## A. List of Abbreviations

AFM	atomic force microscopy.....	6
AHL	acylhomoserine lactone.....	2
AO	acrydine orange.....	6
ATP	adenosine triphosphate.....	7
ATR	attenuated total reflection.....	8
BCE	biofilm collection efficiency.....	24
CLSM	confocal laser scanning microscopy.....	6
CMOS	complementary metal oxide semiconductor.....	28
CPU	central processing unit.....	28
DAPI	4,6-diamidino-2-phenylindole.....	6
DNA	deoxyribonucleic acid.....	4
DTM	differential turbidity measurements.....	7
EDIC	episcopic differential interference contrast.....	6
EPS	extracellular polymer substances.....	2
FAD	flavin adenine dinucleotide.....	9
FISH	fluorescence <i>in situ</i> hybridization.....	12
FOS	fiber optic sensors.....	7
FTIR	Fourier transform infrared.....	8
FWHM	full width at half maximum.....	19
GUI	graphical user interface.....	29
IR	infrared.....	8
JTAG	joint test action group.....	28
LED	light emitting diode.....	19
$\mu$ C	microcontroller.....	28
NA	numerical aperture.....	25



## List of Abbreviations

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NADP <sup>+</sup>	nicotinamide adenine dinucleotide phosphate.....	9
NIR	near infrared.....	8
OCT	optical coherence tomography	
PC	personal computer.....	29
Phe	phenylalanine.....	17
PMT	photomultiplier tube.....	29
PPS	pulsed photoacoustic spectroscopy.....	9
RET	resonance energy transfer.....	17
RISC	reduced instruction set computer.....	28
rRNA	ribosomal ribonucleic acid.....	6
SD	secure digital.....	29
SEM	scanning electron microscopy.....	6
SPI	serial peripheral interface.....	29
SPR	surface plasmon resonance.....	8
TEM	transmission electron microscopy.....	6
Trp	tryptophan.....	17
Tyr	tyrosine.....	17
USB	universal serial bus.....	29
UV	ultraviolet.....	17



## B. Published Patent Application

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(71) Anmelder:

**Christian-Albrechts-Universität zu Kiel, 24118,  
Kiel, DE; IFM-Geomar, Leibniz-Institut für  
Meereswissenschaften an der Universität Kiel,  
24118, Kiel, DE**

(74) Vertreter:

**Jensen & Emmerich, 24941, Flensburg, DE**

(72) Erfinder:

**Friedrichs, Gernot, Prof. Dr., 24808, Jevenstedt,  
DE; Wahl, Martin, Prof. Dr., 24107, Kiel, DE;  
Fischer, Matthias, 24118, Kiel, DE**

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Prüfungsantrag gemäß § 44 PatG ist gestellt.

**Die folgenden Angaben sind den vom Anmelder eingereichten Unterlagen entnommen**

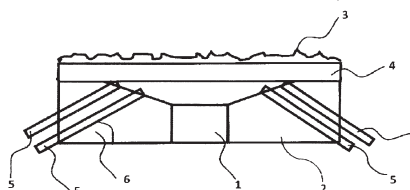
(54) Bezeichnung: **Großflächiger Biofilmsensor**

(57) Zusammenfassung: Die Erfindung betrifft einen optischen Biosensor mit rückwärtiger Beleuchtung zur Detektion großflächiger Biofilme (3).

Der Biofilm (3), der auf dem Siedlungssubstrat (4) direkt auf einer UV-transparenten Oberfläche aus Quarzglas und/oder Plastik siedelt, wird dabei rückwärtig mit geringer Lichtstärke beleuchtet. Durch Änderung des Anstellwinkels (6) der Glasfasern (5) kann zudem der Detektionsbereich der Glasfasern (5) so eingestellt werden, dass im Idealfall nur die Fluoreszenz aus dem Biofilm (3) detektiert wird. Ebenfalls kann dadurch die Mess-Eindringtiefe in den Biofilm (3) variiert werden.

Durch die erfindungsgemäße ringförmige Anordnung der Glasfasern (5) um die schmalbandige Lichtquelle (1) wird gewährleistet, dass möglichst viel Fluoreszenzlicht und wenig Streulicht auf den Detektor geleitet werden.

Mit der Vorrichtung ist es möglich, hoher Anteil der Fluoreszenzemission bei gleichzeitig großer Anregungsfläche des Biofilms (3) einzusammeln und auf einen Detektor zu leiten.



DE 10 2011 101 934 A1 2012.11.22

**Beschreibung**

**[0001]** Die Erfindung betrifft einen Biofilmsensor zur Bestimmung biologischer Eigenschaften eines Biofilms.

**[0002]** Biofilme bilden sich in aquatischen Systemen an nahezu jeder Oberfläche und repräsentieren damit einen Teil der mikrobiologischen Gesamtgemeinschaft. Es entwickeln sich dreidimensionale Strukturen aus Bakterienzellen, extrazellulären polymeren Stoffen und Partikeln. Die Besiedlung einer Oberfläche erfolgt nicht regelmäßig, sondern inhomogen.

**[0003]** Treffen elektromagnetische Wellen von der Wellenlänge einiger Hundert Nanometer auf einen Biofilm, so können diese reflektiert, gestreut und absorbiert werden. Eine möglichst genaue Bestimmung der Besiedlungsdichte wird daher unter anderem dadurch erreicht, dass eine große Oberfläche, möglichst größer als ein Quadratzentimeter, vermessen wird.

**[0004]** Durch die Absorption von Licht im UV-VIS-Bereich lassen sich verschiedene Fluorophore in biologischen Zellen mit elektromagnetischen Wellen zur intrinsischen Fluoreszenz anregen. Als natürliche Fluorophore können eine Vielzahl von Molekülen verwendet und unterschiedliche Parameter gemessen werden. Zum Beispiel ergibt sich für die Messparameter DNA, RNA bei einer Extinktion von 258 nm als Biomasse-Messsignal eine Emission von 320 nm, für Aminosäuren, die bei 280 nm angeregt werden, als Biomasse-Messsignal eine Emission von 350 nm und bei NADH als Messparameter für Stoffwechselaktivität mit einer Extinktion von 340 nm ein Messsignal bei einer Wellenlänge von 460 nm. Durch Messung der Fluoreszenzintensität von Biofilmen kann somit auf die Biomasse, die Stoffwechselaktivität, die Zellzahl und die prozentuale Besiedlungsfläche geschlossen werden.

**[0005]** Es sind unterschiedliche optische Detektionsmethoden zur kontinuierlichen Messung von Biofilmen bekannt, die auf spezifischen Wechselwirkungen zwischen Licht und die den Biofilm aufbauenden Molekülen basieren. Als Beispiel für bisher verwendete Systeme seien unter anderem glasfasergekoppelte Streulichtsensoren – die die unspezifische Rückstreuung des emittierten Lichts messen – und glasfasergekoppelte Fluoreszenzsensoren genannt. Ein spezieller Absorptionssensor ist der ATR-Sensor, der nach seinem Funktionsprinzip – Attenuated Total Reflection – so bezeichnet wird. Weitere Sensoren dieser Art basieren auf der Surface Plasmon Resonance; sogenannte SPR-Sensoren.

**[0006]** Mit ATR- und SPR Sensoren lassen sich sehr empfindlich erste Ansiedlungen von Bakterien messen. Komplexe natürliche Biofilme können mit diesen

Methoden aber nicht quantifiziert werden, da die Eindringtiefe der evaneszenten Wellen in den aufwachsenden Biofilm im Bereich von nur wenigen Hundert Nanometern liegt.

**[0007]** Weiterhin bekannt wurden in den letzten Jahren drei vergleichbare Biofilmsensoren, die in den US-Patenten 6,718,077 B1 und 7,190,457 B2 sowie in der internationalen Patentanmeldung WO 2006/0254343 A1 beschrieben sind. Ein Nachteil der beschriebenen Systeme, die auf Messung der direkten Fluoreszenz mit einer Glasfaser beruhen, ist, dass durch das den Biofilm umgebende Medium hindurch detektiert wird.

**[0008]** Wie bereits oben beschrieben, können Proteine, die aus Aminosäuren bestehen und in Biofilmen zahlreich enthalten sind, durch UV-Licht zur Fluoreszenz angeregt werden. In Proteinen dominiert aufgrund der hohen Absorptionsquerschnitte und Fluoreszenzquantenausbeute in der Regel die Tryptophan-Fluoreszenz. Das Tryptophan weist ein Absorptionsmaximum bei einer Wellenlänge von 280 nm auf und besitzt einen Fluoreszenzemissionspeak bei 350 nm.

**[0009]** Die Eigenfluoreszenz solcher Biofilme kann selektiv durch schmalbandige LED-Quellen oder einer anderen Lichtquelle angeregt werden. Dabei ist es wichtig, auf eine möglichst stabile und gleichförmige Anregung des Biofilms zu achten. Temperaturbedingte Intensitätsschwankungen einer LED als Lichtquelle sollten über eine temperaturkompensierte Konstantstromquelle ausgeglichen werden oder über einen Referenzkanal gemessen werden. Zur weiteren Selektion der Anregungswellenlänge können Bandpassfilter eingesetzt werden. Zum Einkoppeln des Anregungslichts und zur Detektion des emittierten Lichts werden heute bevorzugt Glasfasern oder optische Linsensysteme verwendet.

**[0010]** Ebenfalls Stand der Technik ist es, dass das emittierte Fluoreszenzlicht – bevor es auf den Detektor gelangt – in der Regel durch wellenlängenselektive, optische Bauelemente, wie Bandpassfilter und/oder Monochromatoren geleitet wird. Dadurch werden Streustrahlung und Störfluoreszenz unterdrückt. Im oben genannten Beispiel erlaubt die Kombination zwischen einer schmalbandigen Lichtquelle und optischen Filtern eine spezifische Detektion der Eigenfluoreszenz des Biofilms bei gleichzeitiger Minimierung der Detektion von Hintergrund- und Störstrahlung. Als Detektoren können Sekundärelektronenvervielfacher oder Lawinenfotodioden (APD) verwendet werden. Damit ist es möglich, einzeln einfallende Photonen der Biofilmfluoreszenz über die Zeit aufzusummieren und so die häufig sehr geringe Fluoreszenzintensität zu messen. Es ergibt sich als auswertbarer Messeffekt innerhalb der einzeln untersuchten Biofilme eine nahezu lineare Korrelation

DE 10 2011 101 934 A1 2012.11.22

zwischen Messsignal und Proteinkonzentration beziehungsweise der Bakterienanzahl.

**[0011]** Alle bisher beschriebenen Systeme nutzen zudem Anregungsquellen mit hohen Energiedichten wie Lasersysteme und Hochdruckdampflampen.

**[0012]** Nachteilig am Stand der Technik ist, dass es nicht gelingt, große Flächen bei gleichbleibender Nachweiseffizienz des Detektionssystems zu vermessen. Großflächige Untersuchungen von Biofilmen sind aber besonders wichtig, da eine Besiedlung in praxi nicht homogen verteilt erfolgt. Ebenfalls nachteilig ist, dass ein direktes Anregen der Fluoreszenz im Biofilm nicht unmittelbar über eine gezielte und gebündelte Einkopplung des Anregungslichts auf den Biofilm erfolgt. Weiterhin nachteilig an bisherigen Systemen ist die häufig kleine Detektionsfläche von nur wenigen Quadratmikrometern, die für makroskopisch gültige Aussagen unbefriedigend sind.

**[0013]** Der Erfindung liegt die Aufgabe zugrunde, eine Vorrichtung in Form eines Sensorkopfes zur Messung großflächiger Biofilme mit hoher Sensitivität zu schaffen.

**[0014]** Erfindungsgemäß wird die Aufgabe gelöst durch einen optischen Biofilmsensor zur Bestimmung biologischer Eigenschaften eines Biofilms auf einem Siedlungssubstrat (4), mit:

- einem Sensorkopf (2),
- einer im Sensorkopf (2) angeordneten, ein definiertes Emissionsspektrum ausstrahlenden Lichtquelle (1), und
- wenigstens einem Detektor zum Detektieren von vom Biofilm (3) emittierten Licht,

dadurch gekennzeichnet, dass eine Mehrzahl lichtleitender Glasfasern (5) unterhalb des Siedlungssubstrats (4) ringförmig um die zentral im Sensorkopf (2) angeordnete Lichtquelle (1) angeordnet ist.

**[0015]** In einer bevorzugten Ausführungsform wird die Aufgabe dadurch gelöst, dass die lichtleitenden Glasfasern (5) in einer Mehrzahl von konzentrischen Kreisen angeordnet sind.

**[0016]** In einer weiteren bevorzugten Ausführungsform wird die Aufgabe dadurch gelöst, dass die in Kreisen angeordneten Glasfasern (5) zueinander versetzt angeordnet sind.

**[0017]** Ebenfalls in einer bevorzugten Ausführungsform wird die Aufgabe dadurch gelöst, dass die lichtleitenden Glasfasern (5) wenigstens in Bezug auf eine räumliche Achse in einem Anstellwinkel (6) schwenkbar eingerichtet sind.

**[0018]** In einer weiteren bevorzugten Ausführungsform wird die Aufgabe dadurch gelöst, dass das Siedlungssubstrat (4) UV-Licht durchlässig ist.

**[0019]** In einer bevorzugten Ausführungsform wird die Aufgabe dadurch gelöst, dass der Biofilm (3) von der Lichtquelle (1) rückwärtig bestrahlt wird.

**[0020]** In einer ebenfalls bevorzugten Ausführungsform wird die Aufgabe dadurch gelöst, dass der Biofilmsensor gekennzeichnet ist durch ein der Lichtquelle (1) und den Glasfasern (5) vorgelagert angeordnetes lichtdurchlässiges Siedlungssubstrat (4) zur Besiedlung mit Mikroorganismen auf der der Lichtquelle (1) und den Glasfasern (5) abgewandten Seite des Substrats (4).

**[0021]** In einer weiteren Ausführungsform wird die Aufgabe dadurch gelöst, dass die Lichtquelle (1) ausgeführt als LED, oder als Laser-Diode oder als UV-Lampe ist.

**[0022]** Ebenfalls in einer weiteren Ausführungsform wird die Aufgabe dadurch gelöst, dass der Detektor bzw. der lichtdetektierende Abschnitt eines Detektors eine Lawinenfotodiode (APD) ist.

**[0023]** Die Erfindung wird im Folgenden anhand von zwei Figuren erläutert. Dabei zeigen:

**[0024]** Fig. 1 den Biofilmsensorkopf in der Seitenansicht und

**[0025]** Fig. 2 den Biofilmsensorkopf in der Draufsicht.

**[0026]** Die Aufgabe wird demnach gelöst, indem eine bevorzugt schmalbandige Lichtquelle (1) mit definiertem Emissionsspektrum mittig im Sensorkopf (2) integriert wird, die von ringförmig, besonders bevorzugt in Reihen versetzten Glasfasern (5), umgeben ist. Damit kann ein möglichst hoher Anteil der Fluoreszenzemission bei gleichzeitig großer Anregungsfläche des Biofilms (3) eingesammelt und mittels der Glasfasern (5) auf einen Detektor geleitet werden. Der Biofilm (3), der auf dem Siedlungssubstrat (4) direkt auf einer bevorzugt UV transparenten Oberfläche insbesondere aus Quarzglas und/oder einer direkt aufliegenden Einweg-Plastikfolie siedelt, wird dabei rückwärtig mit geringer Lichtstärke gleichmäßig beleuchtet. Durch die erfindungsgemäß ermöglichte Variation des Siedlungssubstrats (4) lassen sich unterschiedliche Oberflächeneigenschaften, wie Oberflächenspannung und Nährstoffangebot, realisieren.

**[0027]** Durch eine mögliche Änderung des Anstellwinkels (6) der Glasfasern (5) kann zudem der Detektionsbereich der Glasfasern (5) so eingestellt werden, dass im Idealfall nur die Fluoreszenz aus dem Biofilm (3) und nicht die Hintergrundfluoreszenz der umge-

DE 10 2011 101 934 A1 2012.11.22

benden wässrigen Lösung, detektiert wird. Durch die erfindungsgemäße ringförmige Anordnung der Glasfasern (5) um die schmalbandige Lichtquelle (1) wird gewährleistet, dass möglichst viel Fluoreszenzlicht und wenig Streulicht auf den Detektor geleitet werden.

**[0028]** Ebenfalls unterstützend hierfür wirkt die bevorzugt rückwärtige Beleuchtung als auch die Anordnung der Fasern, durch die die Anregung und Detektion von Fluorophoren, die sich in der umgebenden Flüssigkeit befinden, minimiert werden. Die rückwärtige Beleuchtung garantiert zudem, dass eine Beeinflussung des Messsignals durch Bewuchs der Lichtquelle selbst vermieden wird. Insbesondere bei kontinuierlichen Messungen über längere Zeiträume findet ungewollte Akkumulation von Zellen auf der Oberfläche der Lichtquelle selbst statt. In diesen Fällen werden die Messwerte verfälscht und lassen sich auch nicht ohne Weiteres korrigieren.

**[0029]** Durch die Integration des Messsignals über die große Fläche wird eine hohe Empfindlichkeit mit  $< 4 \cdot 10^4$  Bakterienzellen pro Quadratzentimeter erreicht, die somit die Messung von Biofilmen in einer sehr frühen Phase der Biofilmbildung, und somit die Anlagerung erster Bakterienzellen, erlaubt. Eine große Besiedlungsfläche auf dem Substrat (4) ist bei Untersuchungen von Biofilmen (3) besonders wichtig, da die Besiedlung nicht homogen verteilt erfolgt und bei der Besiedlung Randeffekte auftreten können, die bei kleinflächiger Auswertung das Messsignal verfälschen können.

**[0030]** Ein weiterer Vorteil des hier vorgestellten Sensorkopfes gegenüber herkömmlichen ATR- und SPR-Sensoren ist, dass das Anregungslicht mehrere Millimeter in den Biofilm (3) eindringt und es so möglich ist, vielschichtige Biofilme (3) vermessen zu können.

**[0031]** Zwecks Erweiterung der Messanordnung kann durch konstruktive Verkleinerung der einzelnen Baugruppen des Sensors [LED, Filter, Detektor] bei gleichzeitiger Erhaltung einer großen Siedlungsfläche das Gesamtsensorsystem für unterschiedliche Messaufgaben/Zielstellungen angepasst werden.

**[0032]** Statt der Glasfasern (5), die das Fluoreszenzlicht auf einen Detektor leiten, können bevorzugt APD-Elemente als Detektoren für eine direkte Messsignalaufnahme eingesetzt werden. Als Anregungslichtquellen sind, je nach Einsatz- und Messaufgabe, kompakte Lichtemitter-, Laserdioden oder Glasfaser-gekoppelte UV-Lampen mit den gewünschten/erforderlichen Emissionsbereichen in Kombination mit den entsprechenden Spektralfiltern verwendbar. Durch die besonders bevorzugte Verwendung von Mikrofiltern können auch mehrere Lichtquellen in den Sensorkopf (2) integriert werden. Dieses ermöglicht

eine gleichzeitige Mehrparameteranalyse, beispielsweise von Biomasse, Stoffwechselaktivität und Zellenergie. Bei gleichzeitiger Mittelung über eine große Fläche können diese Werte direkt miteinander verglichen werden.

**[0033]** Wird auf eine Anregung des Biofilms (3) verzichtet, können in einer nächsten Erweiterung des Sensors biolumineszente Biofilme [z. B. *Vibrio Fischeri*] auf einer großen Oberfläche untersucht werden.

**[0034]** Durch einen minimalen Einsatz von Baugruppen, einem platzsparenden Aufbau, sowie der geringen Wärmeentwicklung und dem geringem Energieverbrauch kann der Sensorkopf (2) ebenfalls in medizinische Geräte integriert werden.

**[0035]** Eine weitere Verwendungsmöglichkeit ergibt sich durch den Einsatz als Sensor zur Regelung und Steuerung von Prozessen, bei der eine Biofilmentwicklung erwünscht oder unerwünscht ist.

**[0036]** Auch kann die Substratoberfläche mit Aptameren [DNA- oder RNA-Fragmente] als bakterienspezifische Bindestellen beschichtet werden. Bei einem so konfigurierten Biosensor kann die Anhaftung von Bakterien an die Aptamere durch eine veränderte Fluoreszenzintensität detektiert werden.

**[0037]** Der so dargestellte kompakte Sensorkopf (2) ermöglicht eine kontinuierliche Messung der Biofilmdynamik auf einer großen UV-transparenten Oberfläche von mehr als einem Quadratzentimeter – sowohl im Labor als auch unter Feldbedingungen.

#### Bezugszeichenliste

- 1 Lichtquelle
- 2 Sensorkopf
- 3 Biofilm
- 4 Siedlungssubstrat
- 5 Glasfaser
- 6 Anstellwinkel

DE 10 2011 101 934 A1 2012.11.22

**ZITATE ENTHALTEN IN DER BESCHREIBUNG**

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**Zitierte Patentliteratur**

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DE 10 2011 101 934 A1 2012.11.22

**Patentansprüche**

1. Optischer Biofilmsensor zur Bestimmung biologischer Eigenschaften eines Biofilms auf einem Siedlungssubstrat (4), mit:  
– einem Sensorkopf (2),  
– einer im Sensorkopf (2) angeordneten, ein definiertes Emissionsspektrum ausstrahlenden Lichtquelle (1), und  
– wenigstens einem Detektor zum Detektieren von vom Biofilm (3) emittierten Licht,  
**dadurch gekennzeichnet,**  
dass eine Mehrzahl lichtleitender Glasfasern (5) unterhalb des Siedlungssubstrats (4) ringförmig um die zentral im Sensorkopf (2) angeordnete Lichtquelle (1) angeordnet ist.

2. Biofilmsensor nach Anspruch 1, dadurch gekennzeichnet, dass die lichtleitenden Glasfasern (5) in einer Mehrzahl von konzentrischen Kreisen angeordnet sind.

3. Biofilmsensor nach Anspruch 2, dadurch gekennzeichnet, dass die in Kreisen angeordneten Glasfasern (5) zueinander versetzt angeordnet sind.

4. Biofilmsensor nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die lichtleitenden Glasfasern (5) wenigstens in Bezug auf eine räumliche Achse in einem Anstellwinkel (6) schwenkbar eingerichtet sind.

5. Biofilmsensor nach Anspruch 1, dadurch gekennzeichnet, dass das Siedlungssubstrat (4) UV-Licht durchlässig ist.

6. Biofilmsensor nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass der Biofilm (3) von der Lichtquelle (1) rückwärtig bestrahlt wird.

7. Biofilmsensor nach einem der vorhergehenden Ansprüche, gekennzeichnet durch ein der Lichtquelle (1) und den Glasfasern (5) vorgelagert angeordnetes lichtdurchlässiges Siedlungssubstrat (4) zur Besiedlung mit Mikroorganismen auf der der Lichtquelle (1) und den Glasfasern (5) abgewandten Seite des Substrats (4).

8. Biofilmsensor nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass die Lichtquelle (1) ausgeführt als LED, oder als Laser-Diode oder als UV-Lampe ist.

9. Biofilmsensor nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, dass der Detektor bzw. der lichtdetektierende Abschnitt eines Detektors eine Lawinenfotodiode (APD) ist.

Es folgen 2 Blatt Zeichnungen



DE 10 2011 101 934 A1 2012.11.22

Anhängende Zeichnungen

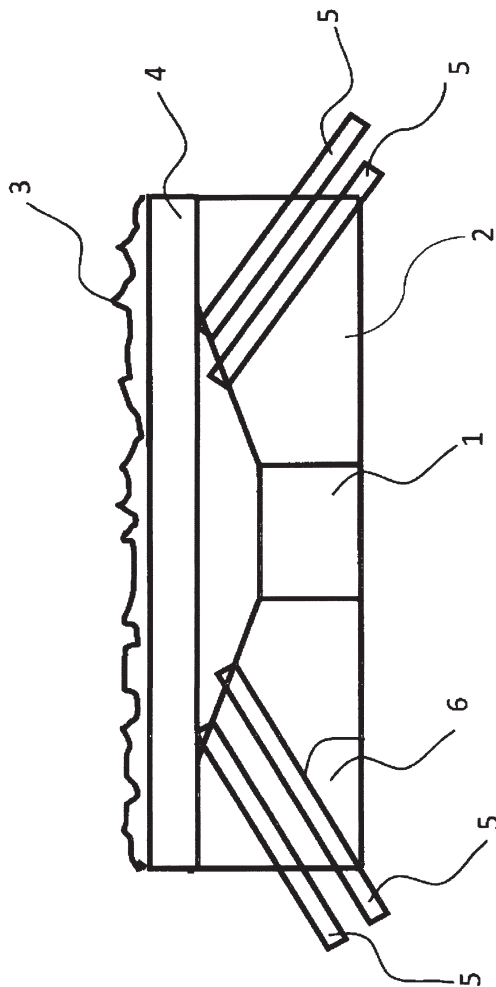


Fig.: 1

DE 10 2011 101 934 A1 2012.11.22

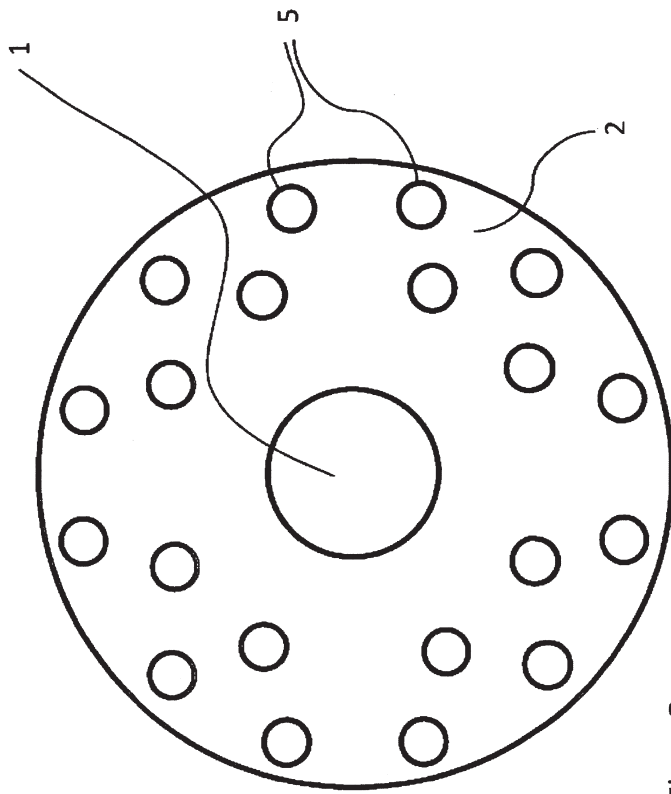


Fig.: 2

## C. List of Publications

Publications included in this thesis:

Paper I:

**M. Fischer**, M. Wahl, G. Friedrichs (2012). Design and field application of a UV-LED based optical fiber biofilm sensor. *Biosensors & Bioelectronics* 33, 172-178.

Paper II:

**M. Fischer**, M. Wahl, G. Friedrichs (2013). Field sensor for *in situ* detection of marine bacterial biofilms. *Sea Technology* 54, 49-52.

Paper III:

**M. Fischer**, G. Friedrichs, M. Wahl. On biofilm dynamics in natural marine environment. (manuscript in preparation for *Applied and Environmental Microbiology*)

Paper IV:

T. Lachnit, **M. Fischer**, S. Künzel, J. F. Baines, and T. Harder (2013). Compounds associated with algal surfaces mediate epiphytic colonisation of the marine macroalga *Fucus vesiculosus*. *FEMS Microbiology Ecology* 84 (2), 411-420.

App. B):

Patent pending: **M. Fischer**, G. Friedrichs, M. Wahl, Großflächiger Biofilmsensor/Large-area biofilm sensor, DE 102011101934.4.

Publications not included in this thesis:

U. Riebesell, J. Czerny, K. von Bröckel, T. Boxhammer, J. Büdenbender, M. Deckelnick, **M. Fischer**, D. Hoffmann, S. Krug, U. Lentz, A. Ludwig, R. Mücke, K.G. Schulz (2012). A mobile sea-going mesocosm system: New opportunities for ocean change research. *Biogeosciences*, 9 (9), 12985-13017.

K.G. Schulz, R.G.J. Bellerby, C.P.D. Brussaard, J. Büdenbender, J. Czerny, A. Engel, **M. Fischer**, S. Koch-Klavsen, S. Krug, S. Lischka, A. Ludwig, M. Meyerhöfer, G. Nondal, A. Silyakova, A. Stühr, U. Riebesell (2012). Temporal biomass dynamics of an Arctic plankton bloom in response to increasing levels of atmospheric carbon dioxide. *Biogeosciences*, 9 (9), 12543-12592.